"ROLE OF VITAMIN D RECEPTOR GENE Taq 1 POLYMORPHISM IN RECURRENT UROLITHIASIS"

Dissertation submitted to

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for the award of the degree of

M.Ch (UROLOGY)

BRANCH IV

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY
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DECLARATION

I solemnly declare that this dissertation titled **"ROLE OF VITAMIN D RECEPTOR GENE Taq 1 POLYMORPHISM IN RECURRENT UROLITHASIS"** was prepared by me in the Department of Urology, Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai under the guidance and able supervision of **Prof. R. Jeyaraman MS, M.Ch.,** Professor & Head of the Department, Department of Urology, Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai. This dissertation is submitted to the Tamil Nadu Dr. MGR Medical University, Chennai in partial fulfilment of the university requirements for the award of the degree of M.Ch. Urology.

Place: Chennai

Date: **Dr. S. Ganesh Prasad**

CERTIFICATE

This is to certify that the dissertation titled **"ROLE OF VITAMIN D RECEPTOR GENE Taq 1 POLYMORPHISM IN RECURRENT UROLITHASIS"** submitted by Dr.Kanagasabapathi M. appearing for M.Ch. (Urology) degree examination in August 2013, is a bonafide record of work done by him under my guidance and supervision in partial fulfilment of requirement of the Tamil Nadu Dr.M.G.R.Medical University, Chennai. I forward this to the Tamil Nadu Dr.M.G.R.Medical University, Chennai.

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INTRODUCTION

Urinary tract stone formation is multifactorial. Alteration in metabolism are found in 40%-60% of stone formers. Among them idiopathic hypercalciuria is the most frequent.

Idiopathic hypercalciuria may be due to

- An increase in intestinal calcium absorption
- Enhanced demineralization of bones

1,25 $(OH)_2$ D_3 regulates target tissue biological response through genomic events which involves steroid hormonal intra cellular Vitamin-D receptor.

Researches has proven that increased calcium absorption is mediated by an increase in number of Vitamin-D receptors in intestine of genetic hypercalciuric rats.

Very little is known about genetic factors that mediate susceptibility to calcium nephrolithiasis. A positive family history is the most important risk factor when dietary factors are ruled out. The identification of genetic factors for calcium nephrolithiasis will lead to understanding of how the disease develops and assisting in evaluation of high risk individuals.

It is proven that in DNA sequence of human genome, many genes are polymorphic. In a specific gene there may be a single base pair substitution of one nucleotide (SNP) for another or valuable number of repeats of a short repetitive DNA sequence of a specific gene. Thereby

- Gene transcription rate may change
- There may be a change in the stability of messenger RNA
- Activity and quality of resulting protein may vary

Thus a number of disorders including nephrolithiasis is influenced by position of specific alleles in polymorphic genes.

Calcitriol, the active metabolite of Vitamin-D has several major roles in the body.

- It regulates the calcium and phosphate homeostasis
- It regulates the synthesis of parathormone
- Immune system and endocrine modulator
- Plays against tumor development by its anti proliferative effect.

Effect of Vitamin-D are mediated via nuclear Vitamin-D receptor. This receptor heterodimerises with retinoid X-receptor and changes gene transcription. So far in literature about 5 single nucleotide polymorphism of Vitamin-D receptor has been reported.

Several polymorphisms have been identified in VDR gene. The functional importance and effects on disease have been investigated

The SNP sites most frequently reported have been

- Start codon (Fok-I)
- Intron 8 (Bsm-I)
- Exon 9(Taq 1)

In Western and Japanese population there is lack of data regarding allelic variations in VDR gene (Taq1). From stone formers in Indian subcontinent where ethnicity is quite different , there is only one such study published from North Indian population. To our knowledge no study has examined the relationship between Vitamin-D receptor gene (Taq1) polymorphism in South Indian population. Therefore the present study is an attempt to investigate it further using a PCR based restriction analysis in unrelated stone forming individuals from our institution belonging to local ethnicity.

AIMS AND OBJECTIVES

The aim of present study is to determine the role of Taq1 polymorphism of Vitamin-D receptor gene in calcium urolithiasis.

Objectives

1. To determine the risk attributed by Vitamin-D receptor polymorphism with specific reference to Taq1 genotype.
2. To perform a stratified analysis of the genotype with clinical characteristics of patients like family history, recurrence, hypercalciuria in them.

REVIEW OF LITERATURE

History of Urinary Stone Disease

Urinary stone disease has been causing concern for more than a million years. The oldest renal stone recorded was by Shattock in an Egyptian mummy in a tomb dating to approximately 4400 BC. Though stone disease was investigated for over a thousand years, scientific research with clear cut investigations determined the stone composition only within the past 200 years.

Hippocrates (460–370 BC), included a passage explaining the treatment of stones in Hippocratic Oath:

"I will not cut persons laboring under the stone, but will leave this work to be done by men who are practitioners of this work."

Epidemiology of renal stones:

Urinary tract stone disease affects about 10% of population in the industrialized part of the world. The annual incidence of kidney stones may go upto 0.6 to 2.0 % in developed nations. In a country like India there are always wide regional variation. Southern part of India has a relatively lower incidence when compared to compared to other parts.Maharashtra has a relatively higher incidence in India(about 7.6%). Equally high incidence has

been reported in Rajasthan and other western parts of India. Various studies from south India estimated the incidence to range between 5 – 7%. In our country 75% of urinary stone disease is due to calcium oxalate stones.

Factors Affecting nephrolithiasis:

Urolithiasis is associated with many complicated factors. It can be triggered by various environmental influences, metabolic defects and genetic factors [1, 2]. The tendency of stone formation is mainly attributed to excessive calcium absorption, since calcium is the principal crystalline constituent in up to 80% of kidney stones [2].

Gender

Adult men are affected two to three times more commonly than adult women . But recent studies show that the difference between the sexes is narrowing quickly. Stamatelou and colleagues and reported such phenomenon in the recent dataset.

Race/Ethnicity

Racial differences has a strong influence with stone disease. The difference in sex ranged from a male to female ratio of 2.3 in western world to 0.65 in Africa while the same ratio is about 3.4 in Indian subcontinent(Soucie et al 1994).

Age:

Peaks during third to sixth decade. World over there are too many variations in various literature. But females in menstrual age group have a protective role played by oestrogen which increases urinary citrate excretion and decreases calcium excretion in urine. (Heller et al, 2002).

Geography

Usually the incidence is high in tropical, hot and hilly regions like mountains and deserts where hot and arid ,dry climates prevail.

Climate

Summer seasons see more stone cases due to two main reasons. One is due to dehydration following perspiration and the other factor is due to increase in production of Vitamin D due to excess sun exposure.

Body Mass Index and Weight:

Increase in body mass has a direct correlation with increase in chance of stone formation. Hypocitraturia and hyperuricosuria are said to be the main reasons.

Water

Incidence of stone disease is inversely related to fluid intake proven by several large observational studies.

Calcium Stones

Hypercalciuria:

Among calcium stone formers the most common abnormality is idiopathic hypercalciuria.

Hypercalciuria is common in 35% to 65% stone-forming of patients have identifiable hypercalciuria as an abnormality.

Various researches done in stone-forming rats having hypercalciuria has clearly suggested that supersaturation of calcium phosphate and hypercalciuria are the major problems in stone formation.

Recently, it's proven that the number of Randall plaques which are the potential precursors of the stone disease correlate with the urine calcium level and the number of stone episodes.

Daily amount of calcium filtered by the kidney is 260 mmol in which only 4 mmol is excreted out in urine, rest all are absorbed back by nephron. Increased urinary calcium forms complexes with chondroitin sulphate and citrate which are actually inhibitors of stone formation especially crystallisation.

Hypercalciuria is clearly defined by Parks et al that the urinary excretion of more than 4 mg/kg/day or more than 7 mmol/day.

Traditionally, the term *idiopathic hypercalciuria i*s a specific term used to denote stone formers whose actual metabolic abnormality was known.

There are three main sites where calcium homeostasis is actually regulated: bone, intestine, and kidney. Dysregulation at any of these sites can lead to hypercalciuria. Genetic hypercalciuric rat models have proven that hypercalciuria may be due to problem with multiple calcium transporting mechanisms. Classically Pak et al classified hypercalciuria into three types: Increased intestinal absorption produces absorptive hypercalciuria , Increased renal leak of calcium produces renal hypercalciuria, unwanted excess bone demineralisation produces resorptive hypercalciuria.

Absorptive Hypercalciuria.

By definition Absorptive hypercalciuria is increased urinary calcium ion excretion after an oral load of calcium. Sometimes even fasting hypercalciuria is seen in this scenario.

Here the pathology is increased calcium absorption from gut.This disease has two type where in type I urinary calcium levels remains high inspite of dietary calcium restrictions, Type 2 where calcium levels in urine is reduced with a calcium restricted diet.

The calcium absorption from the gut suppresses PTH leading to increase calcium leak in urine.Since increased calcium absorption is matched by excess calcium loss in urine these patients have a normal calcium level inspite of increased absorption.

Although there is no clear cut mechanism to explain increase in calcium absorption from gut,the proposed mechanisms shift the focus to an alteration in the vitamin d gene receptor in intestine.

Some others suggest that hyper sensitivity to Vitamin D by the receptor may be the cause. Hypercliuria has been investigated and linked to Vitamin D receptor by many genes.In study conducted by Jackman and colleagues(1999) Vitamin d gene polymorphism was linked to positive family history of stone disease. Also, The link between microsatellite marker and VDR gene focus on chromosome 12 q with calcium stone disease has been investigated by Scott and colleagues .

There are so many other studies too which fail to establish clear link between VDR gene focus and nephrolithiasis. Further researches showed a genetic locus for Absorptive hypercalciuria linked to chromosome 1q.

There are other metabolic abnormalities like the Hereditary Hypophosphataemic rickets in which excess phosphate waste in urine triggers vitamin D activity leading to increased calcium absorption from gut and

thereby excess calcium excretion in urine. This disease is inherited in an autosomal recessive pattern. R *SLC34A1* and *SLC34A3 are the responsible genes.* NaPi-IIa and NaPi-IIc are the sodium phosphate transporters affected commonly in this disorder. But this disorder of Hypophosphataemic Hypercalciuric Rickets is an extremely rare disorder accounting for just 2% of patients.

Renal Hypercalciuria.

98% of calcium filtered by glomerulus is absorbed back by the kidney.70 % absorbtion takes place in proximal convoluted tubule. Though there may be renal loss of calcium thus is compensated aptly by the combined action of two hormones PTH and Vitamin D which act together in maintaining in serum calcium levels.

So this disorder has a high level of urine calcium in spite of having normal serum calcium. This increased fasting urinary calcium and elevated PTH levels distinguish renal and absorptive hypercalciuria.

There are direct and indirect evidences to show the renal calcium leak. Sodium cellulose phosphate which is a calcium binding resin is not useful in them and urine calcium stays high inspite of taking it. Secondly there is an exaggerated response to hydrochlorthiazide diuretic ,since the problem is at the

renal tubular level.Calciuric response to oral glucose test is another test to predict renal hypercalciuria.

Various theories have been proposed to explain this issue.

Defect in chloride channel CIC-5 is ssen in diseases such as Dent disease and X linked hypophosphataemic rickets leading to hypercalciuria ,nephrolithiasis,proteinuria. This transmembrane chloride channel deals with transmembrane voltage potential homeostasis.

Abnormality in chloride channel leads to hypercalciuria by activating Vitamin D indirectly.Anyhow, the exact mechanism is clearly not known.

Mutational changes in gene producing calcium sensing receptor usually leads to decreased serum calcium levels and excess calcium loss in urine not attended by action of PTH as the calcium sensing receptors are naive.This produces a state of salt losing nephropathy and ultimately leads to dysfunction of apical potassium channel ROMK.

Reported are disorders of sodium potassium chloride channel(Na+K+2Cl-) and individual potassium channel receptors(ROMK) which are reported to produce hypercalciuria. Basically these channel disorders are autosomal recessive disorders.

An autosomal recessive disorder associated with such channels is given a special name called Bartter syndrome which produces hypercalciuria, hypokalemia and secondary hyperaldosteronism.

Genetics factors attributing to calcium nephrolithiasis:

To understand the mechanism producing calcium stone formation and to know how the calciurics respond to drug, Genetic analysis is required. Mainly Single-candidate gene polymorphisms is looked as an researchable area in stone formers. In the era of having an idea of whole genome ,it may be possible to work out the risk of an individual to develop a stone ,how to treat them and advising them adequate diet which will prevent future stone disease. Twin and family studies has proven the importance of calcium nephrolithiasis. The relatives of stone forming patients always have a higher incidence stone formation when compared to non relatives.(1,3) Similarly monozygotic twins have a higher incidence when compared to dizygotic twins(5).

A GENETIC APPROACH TO UROLITHIASIS:

Based on current family based disequilibrium studies, responsible genes were tested by means of case control or cohort analysis.

There may be tests that may look for single nucleotide polymorphism or a genetic marker pertaining to a particular trait is isolated and these tests are known as linkage dis equilibrium tests. So, an interested phenotype can be

mapped to a particular loci thereby mapping most of monogenic disorders on genes(6).

Case control study is often used to identify the allelic distribution of desired characters in a set of genetically comparable individuals.

So, always there is a possibility of falsely claiming a phenotypic character without a true association because of the phenomenon of genetic heterogenicity (7).

Apart from it, every gene also keeps interacting with other genes and environment which may influence the outcome of the disorder/phenotype (epistasis)(7,8).That is the reason why most of these assosiations reported from these studies cannot be repeated in another ethnic group.So, most of these studies require a huge sample population in order to stratify people according to phenotypic and genetic back ground.

Calcium salt precipitation or calcium metabolism altering genes producing calcium nephrolithiasis has been studied detaily. 1alpha-hydroxylase of the 25(OH)-hydroxyvitamin D (VDD1), vitamin D receptor (VDR),Calcium sensing receptor(CASR), uromodulin (UMOD), osteocalcin (OCG), and osteopontin (OPN) were all tested for linkage dis equilibirium for stone formation(8,9,10). VDR gene locus (12.q12-14) was the only one among all these studies to show some direct association.

Micro satellite markers were traditionally used in addition to restriction fragment length polymorphism in locating the exact foci of responsible culprit genes which produce calcium nephrolithiasis (9). These studies were positive for six of indian families whereas nine of European families didn't show any association.(11.12).

STUDIES ASSOCIATED WITH SINGLE-CANDIDATE GENES:

Of recent interests is various studies which link calcium nephrolithiasis and hypercalciuria to VDR gene polymorphism sites worked up with specific Restriction fragment length polymorphisms in either exon 9 site Taq I or intron 8 site enzyme like BsmI,Apa I, or starter codon enzyme of FokI.(13,14)

Early onset, higher frequency of stones and decreased urinary citrate excretion were explained with allelic variations(13.15).Altogether the protein produced by VDR gene polymorphic variants is not going to be different but the actual change will be in mRNA stability of the product which is the actual proposed mechanism of alterations in urinary calcium excretion among various polymorphs (16).

The enzyme that mediates the absorption of citrate in the proximal tubular cells ie., phosphoenolpyruvate carboxykinase is altered by different polymorphic varieties of VDR gene which may be the reason for

hypocitraturia that can produce calcium nephrolithiasis by an indirect means (17)

SUMMARY OF FINDINGS IN GENETIC RESEARCHES IN NEPHROLITHIASIS:

Though the actual weightage and pathogenic impact on calcium nephrolithiasis by various genes like VDR ,CLDN14, CASR and OPN is not clearly known researches are on theirway to pinpoint the location for type of insult producing human calcium nephrolithiasis.

Polymorphisms of Vitamin D Receptor gene ,Calcium Sensing Receptor Gene and chloride channel gene(CLDN14) genes were implicated in producing calcium nephrolithiasis:

Calcium sensing receptor gene is usually associated with normocitraturic hypercalciuia calcium nephrolithiasis.

VDR gene polymorphisms is associated with hypocitraturia ,strong family history of calcium nephrolithiasis.

VITAMIN D - GENERAL PHYSIOLOGY AND FUNCTION:

Normal bony development with adequate mineralization of a skeleton is based on action of the “Hormonal Vitamin” Vitamin D.The action of Vitamin D is carried on intestines and bone by means of Vitamin D receptor but , to

everybody's surprise this receptor is located not only in intestine and bone but every cell of our body.

Daily requirement of this vitamin is adequately obtained by sun exposure. Thus endogenously produced vitamin D will be sufficient for calcium homeostasis. In addition to it people who are deficient in this can also receive an oral supplementation in the form of vitamin D3 (cholecalciferol) or D2 (ergocalciferol, derived from the irradiation of plant sterols). Cod liver oil is a very good source of Vitamin D and apart from it most of fish products.In European countries most of food stuffs like milk, orange juice, and some cereals, breads, yogurts, and cheese are fortified with vitamin D.

The conversion of 7 dehydro cholesterol to previtamin D3 occurs secondary to ultraviolet B light (wavelength 290-315 nm) exposure and this happens in epidermis and dermal layer. In Body heat Vitamin D3 forms from previtamin D3 by means of isomerization.Vitamin D3 in turn binds to vitamin D-binding protein (VDBP) in serum.

In presence of 25 hydroxylase of liver Vitamin D3 is converted to 25(OH) D3 .In human serum 25(OH) D3 is the major vitamin D metabolite and it's known as the best indicator of individual Vitamin D status .

1-alpha hydroxylase present in kidneys convert 25(OH) D3 to 1,25-dihydroxyvitamin D3 [calcitriol] by hydroxylating it. Calcitriol is the most

biologically active form of Vit D 3. 1,25 dihydroxyvitamin D3 mainly has it's action in the intestine, kidney, and bone to regulate calcium and phosphate metabolism.

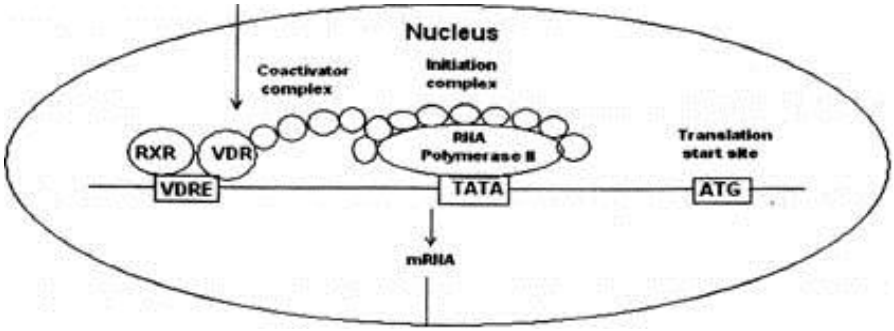
VITAMIN D RECEPTOR (VDR)

It's a nuclear receptor belonging to steroid hormonal family. Also known as calcitriol receptor. Ultimately vitamin D acts through this nuclear receptor and produces new gene expression by mediating necessary protein synthesis. It is encoded on chromosome 12.

VDR contains 427 amino acids which has a ligand binding domain,two zinc fingers,a flexible hinge region, a DNA binding domain and an activation function domain.The hinge region has a Heat Shock Protein 70 site.The ligand binding domain has a C- terminal with a transcriptional activation function.

Structure of Vitamin D receptor

Calcitriol enters the cell and binds to VDR, after which the complex is phosphorylated. This complex binds to retinoic acid or retinoid X receptor. Vitamin D response elements get attached to the zinc fingers on VDR. Approximately 60 genes are regulated by Vitamin D. That's why even a small mRNA transcriptional change in the receptor usually brings major changes in so many genes produced.



Role of Vitamin D in regulation on gene expression

Intestinal calcium channels and calcium binding proteins are upregulated by the action of calcitriol. This favours calcium transport from lumen to circulation. Similar receptor are also found in osteoclasts of bone which help in absorbing calcium from boine in times of need to maintain the calcium homeostasis.

There are specific signal pathways which have an influence on the positive and negative feedback mechanism to regulate the activity of 1,25-dihydroxyvitamin D3 both in intestines and bones. Apart from intestine and bone, 25(OH)D-l-hydroxylase is also present in so many other tissues thereby favouring these tissues to produce their own active hormones at times of need. Eg. Prostate, colon, lung, breast.

VITAMIN D RECEPTOR GENE

The human VDR is exactly located at chromosome 12ql3-14. Studies on osteoporotic females turned the attention of the world towards this gene which led to further research on bone mineral density and calcium

homeostasis. The ligand binding region is coded by exons 4 to 9,while exon 2 and 3 code for a DNA binding domain, whereas exons IA and IF cod ethe untranslated region in 5' terminal and altogether there are 14 exons.

SINGLE NUCLEOTIDE POLYMORPHISM (SNP)

If there is a difference between paired chromosomes or when there are variations in a single nucleotide among members of a same species this DNA sequence variation is called single nucleotide polymorphism.

To define an entity as a SNP it has to occur at least in 1% of population. SNP usually predicts the risk of an individual to get particular disease like renal or heart disease, how they respond to medical treatment..The SNP pattern found in affected individuals is compared with the SNP pattern of an unaffected individuals to determine the level of association of specific disease. Disease susceptibility to one particular disease process for a particular SNP can be fairly judged if that SNP is common in affected population.

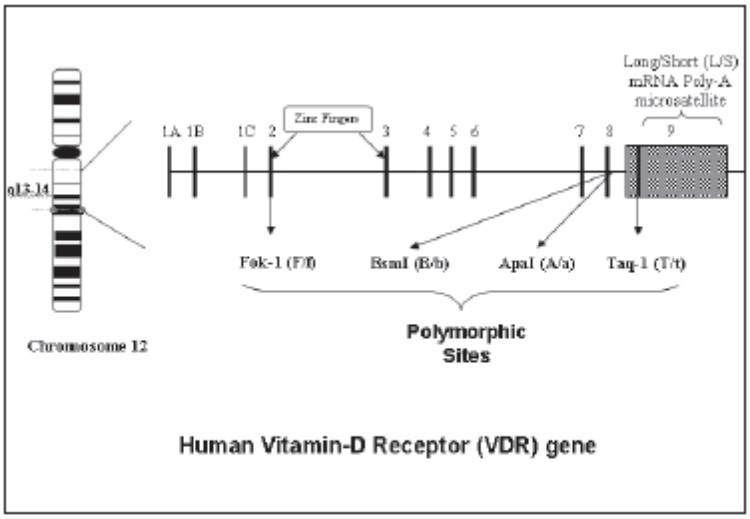
VITAMIN D RECEPTOR GENE POLYMORPHISMS

Most studies of Vitamin D receptor and calcium urolithiasis have focused on five polymorphisms:

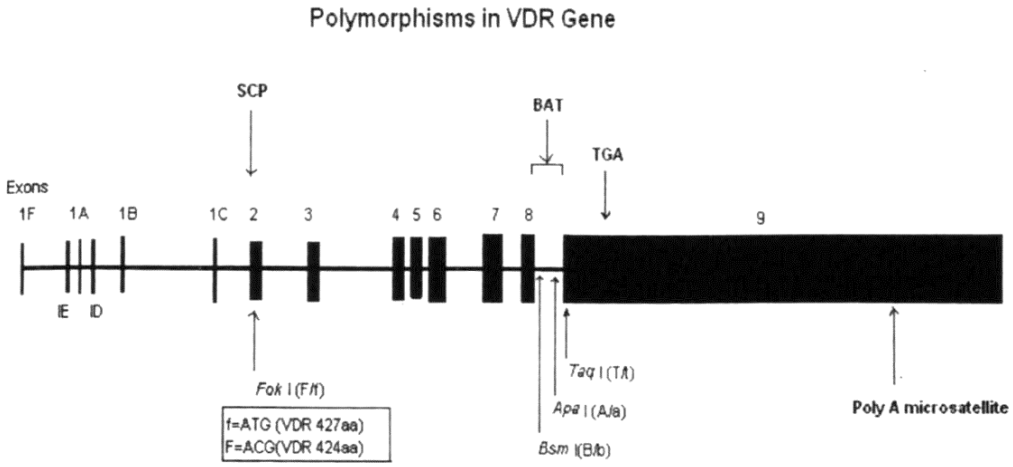
1) rs 10735810 or Fokl polymorphism on exon 2 (F and f)
2) rs 1544410 or BsmI on intron 8 (B and b)
3) rs 731236 or TaqI on exon 9 (T and t)

4) rs 7975232 or ApaI on intron 8 (A and a)

5) poly(A) mononucleotide repeat at the 3-UTR section of the gene. (L and S)



Schematic diagram of VDR gene demonstrating different restriction sites



The function of product genes mainly vary based on the location of genetic polymorphism. Therefore the start codon polymorphism may produce an effect totally different from the one located near ligand binding domain.

When specific alleles tend to be linked together it's known Linkage disequilibrium. There is also proven fact that poly A alleles in 3' UTR region are in linkage disequilibrium with Bsm I, ApaI and Taq I.

These genetic variations may not actually correlate well with change in Vitamin D Receptor but actually has it's influence on VDR responsive gene expression. For example "t" allele of TaqI has higher activity when compared to "T" allele. Similarly "S" allele of Poly A polymorphism has similar higher activity when compared to "L: allele.

Most studies of VDR and calcium urolithiasis have been conducted predominantly in non-Hispanic white populations. More studies are needed in other races. To our knowledge, no studies have examined the relationship between Vitamin D receptor gene polymorphisms and risk of urolithiasis in South Indian population.

It's quite common to find allelic variations seen in VDR gene in various populations. Thymine/cytosine polymorphism in first two start codons Separated by 3 codons is a known DNA sequence variant.

Restriction fragment Length Polymorphism is used to differentiate various alleles in Fok I ,Apa I ,Bsm I,and our gene of interest Taq I.

Taq*I* *Polymorphism in urolithiasis studies so far:*

So far 9 studies have been reported in literature.When all of the 9 studies were pooled, no significant study heterogeneity was detected . Marginal positive association was found between the *Taq*I polymorphism and urolithiasis risk in allelic comparisons, while the pooled analyses in the dominant model and recessive model were insignificant. But, sensitivity analysis after excluding all the studies with controls deviating from Hardy Weinburg Equilibrium indicated an increased urolithiasis risk associated with tt+Tt genotype in the dominant model (OR = 1.35, 95% CI).

Stratified analysis based on the regional characteristics suggested a similar significant association in the dominant genetic model in the Asian subgroup (OR = 1.41, 95% CI) but the other subgroups yielded insignificant results. Moreover calcium stone-specific associations also yielded insignificant results.

There are various studies based on SNP sites like start codon FokI ,Intron 8 BsmI polymorphism and calcium urolithiasis in European and Asian ethnicity. However, there are no reported studies till date which analyses the role of Taq I polymorphism and its association to calcium nephrolithiasis from

the ethnicity belonging to South Indian population. PCR based restriction analysis is used to analyse VDR gene Taq I polymorphism and its role in stone formation, exclusively based on south Indian population.

MATERIALS AND METHODS

Subjects:

50 patients being treated in Urology Department of Rajiv Gandhi Government General Hospital between age group 20 to 70 years with documented calcium stone disease with a history of spontaneous stone passage or any kind of treatment for stone disease whose stone composition had calcium as major component were included in the study.

Of these 50 patients, 25 had only one episode of calcium-stone disease and they were stratified as Group 1 and the remaining 25 patients with recurrent calcium-stone disease constituted Group 2 . Fifty normal subjects with no history or radiological finding of stone disease composed the non-stone former control group Group 3.

Inclusion criteria ;

Calcium containing renal stone detected and treated where calcium being the major component of the calculus/calculi.

Exclusion criteria:

1. Any patients with the history of medications that affect urinary calcium excretion or excessive intake of vitamin D or excessive calcium intake were excluded.

2. Patients or controls with abnormal Vitamin D, Calcium or Phosphorous serum levels were also excluded from the study.
3. Infection stones(struvite stones) or stones due to congenital enzymatic or metabolic abnormality were all excluded from the study.

Family History:

A family history of urolithiasis was sought from each patient. Family history was considered positive if any of sibling, a parent, grandparent or parental siblings had a history of renal-stone disease.

The study was performed with local Ethical Committee approval, and informed consent was obtained from all subjects.

Blood samples for measurement of biochemical parameters were obtained in the fasting state at 8 AM. Serum calcium and phosphate were measured with the use of AutoAnalyzer. Serum concentrations of Vitamin D3 was measured by radioimmunassay method (RIA).

Hypercalciuria is defined by a 24-h urinary calcium excretion more than 4 mg/kg per day . The 24-h urinary calcium excretion results were available in all 100 subjects(Both cases and controls) comparison between genotypes were carried out accordingly.

Groups were compared in terms of age, gender distribution, presence of familial (first degree) stone disease history and the status of VDR Taq I gene polymorphism.

GENOMIC DNA ISOLATION BY HIGH SALTING METHOD

MATERIALS REQUIRED:

- Red Cell lysis buffer (RCLB)

  (Tris HC1 - 10 mM, Magnesium Chloride - 10 mM,

  Potassium Chloride - 10 mM, EDTA - 2 mM)

- Nucleated Cell lysis buffer (NCLB)

  (Tris HC1 - 10 mM, Magnesium Chloride - 10 mM, Potassium

  Chloride - lOmM, EDTA - 2 mM, Sodium Chloride – 400 mM)

- Triton X 100
- Sodium dodecyl sulphate - 10%
- Sodium Chloride - 5M
- Absolute Ethanol
- 70% Ethanol
- Sterile water

PROCEDURE:

RBC lysis

The peripheral blood samples from the vacutainer (3-4 ml) containing EDTA was taken in labeled polypropylene tubes. Double the volume of Red cell lysis buffer (RCLB) and six drops of 0.1% commercially obtained Triton - X 100 was added to the blood, mixed gently and incubated in a water bath at 37°C for 5 minutes.

Tubes were removed from the water bath and centrifuged at 2000rpm, 4°C for 15 minutes. The supernatant was discarded carefully without disturbing the pellet. To the pellet, 10ml of RCLB was added and vortexed gently and centrifuged at 2000rpm, 4°C for 15 minutes. The supernatant was discarded carefully without disturbing the pellet.

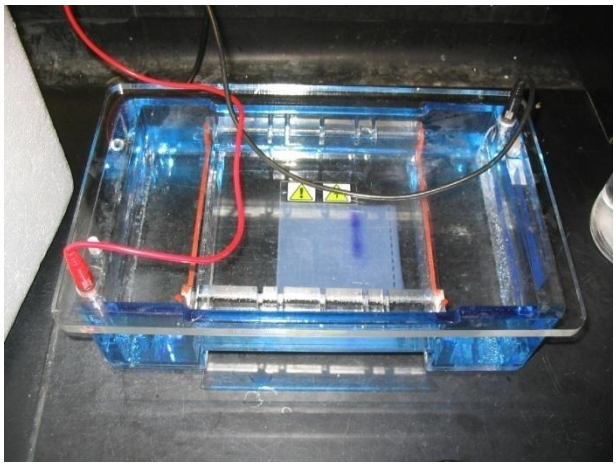
WBC lysis

To the pellet, 1 ml of Nucleated cell lysis buffer (NCLB) and 20ul of 10% SDS was added and vortexed._The tubes were incubated at 55°C for 1 hour. At the end of 1 hour, the contents were transferred into a microfuge tube and 400 ul of 5M Sodium Chloride was added. The tubes were centrifuged at 10000 rpm, 4°C for 15 minutes.

DNA precipitation

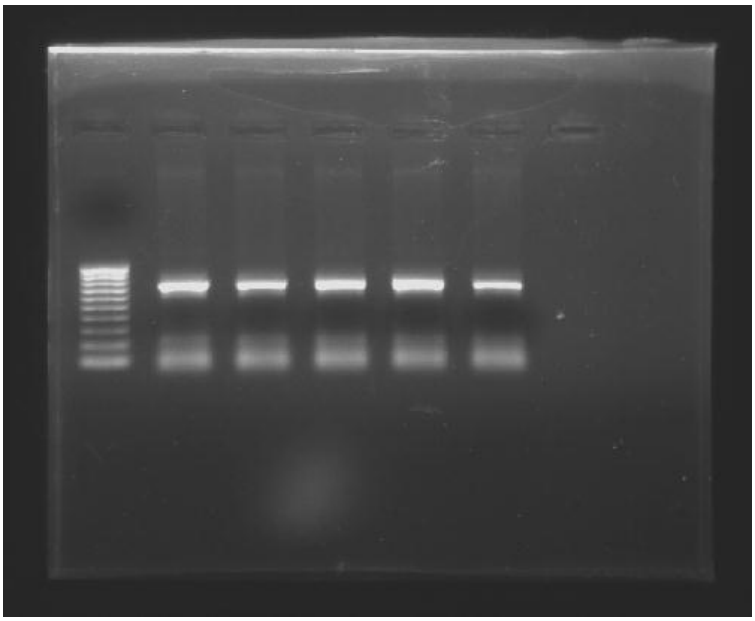
The supernatant was transferred into a polypropylene tube and added double the volume of chilled absolute ethanol. The visible DNA strands were transferred into a labeled microfuge tube with a micropipette and 400 ul of 70% ethanol was added. The tubes were centrifuged at 2000 rpm, 4°C for 5 minutes. The supernatant was discarded and pellet was allowed to air dry. The pellet was dissolved in 100 - 150 ul of sterile nuclease free water and incubated at 4°C overnight for dissolving the DNA.

AGAROSE GEL ELECTROPHORES



GEL DOCUMENTATION SYSTEM:

DNA VISUALISED ON GEL



POLYMERASE CHAIN REACTION

MATERIALS REQUIRED

- Forward primer
- Reverse primer
- lOXTaq buffer
- Taq polymerase enzyme (3U/ul)
- Deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) - 10 mM
- DNA samples (control and patients' DNA samples) - 100 ng
- Sterile water

SEQUENCE OF THE PRIMERS

The primer sequences for TaqI polymorphisms:

- 5′-GGGACGATGAGGGATGGACAGAGC-3′ and
- 5′-GGAAAGGGGTTAGGTTGGACAGGA-3′.(51)

PCR REACTION MIXTURE:

S. No	COMPONENTS	CONCENTRATION	QUANTITY(ul)
1.	Sterile water		14.5
2.	10X Taq buffer	IX	2.0
3.	dNTPmix	0.2 mM	0.4
4.	Forward primer	50 pmoles	0.3
5.	Reverse primer	50 pmoles	0.3
6.	Taq Polymerase	1.5 units	0.5
7.	DNA samples	100 ng	2.0

GRADIENT PCR FOR STANDARDIZATION OF ANNEALING TEMPERATURE

The above components were added to 0.2 ml PCR vials and Gradient PCR was performed for a gradient of 55°C to 65°C to standardize the annealing temperature. 10 ul of the PCR products thus obtained were mixed with 2 ul of 6X gel loading dye and electrophoresed on a 2% agarose gel containing EtBr, (0.5 ug/ml) along side a lOObp marker (Bangalore Genei). The optimum annealing temperature for Apal and TaqI was 61°C and for BsmI was 63°C.

PCR CONDITIONS FOR VDR GENE:

(EXON 9 TaqI)

CONDITIONS	TEMPERATURE	TIME	TEMPERATURE	TIME
Initial denaturation	94°C	5 mins	94UC	5 mins
Denaturation	94°C	45 sees	94UC	45 sees
Annealing	61UC	45 sees	63°C	30 sees
Extension	72°C	1 min	72°C	1 min
Final extension	72°C	5 mins	72°C	5 mins

Total number of cycles = 30. Hold at 4 °C after 30tn cycle

PURIFICATION OF PCR PRODUCT

MATERIALS REQUIRED:

PCR product - 20 ul Absolute ethanol

Nuclease free sterile water - 70% ethanol

Sodium acetate (pH - 5.2) - 3M

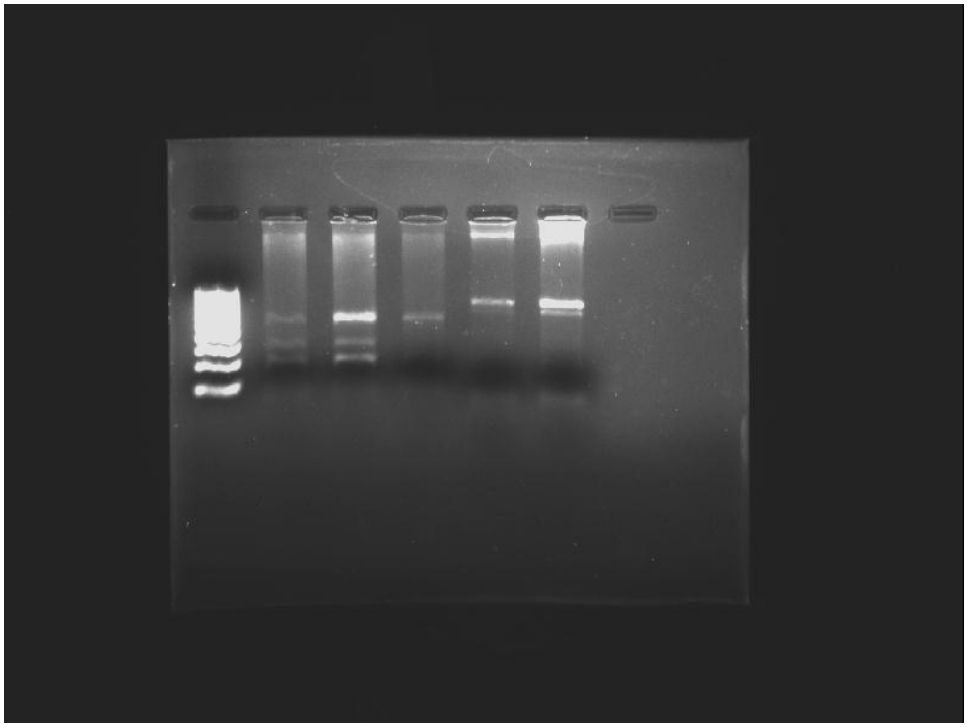
PROCEDURE:

The PCR product (20 ul) was transferred into a microfuge tube and triple the volume of nuclease free sterile water (60 jul) and one-tenth the volume of 3M Sodium Acetate (pH - 5.2) was added. To this mix, double the volume of Absolute ethanol was added and kept in freezer overnight for precipitation. Next day, the microfuge tubes were centrifuged, at 10000 rpm for half an hour at 4°C.

Supernatant was discarded and 200 ul of 70% ethanol was added to microfuge tubes and centrifuged, at 10000 rpm for half an hour at 4°C. Supernatant was discarded immediately and the microfuge tubes were air-dried. 10 ul of sterile nuclease free water was added to the microfuge tubes and the purified product was allowed to dissolve overnight in the refrigerator.

PCR ANALYSIS

RFLP ANALYSIS OF TaqI IN AGAROSE GEL:



RESTRICTION FRAGMENT LENGTH POLYMORPHISM

REACTION MIXTURE:

S.No	COMPONENTS	WORKING CONCENTRATION	QUANTITY ADDED(uL)
1.	Purified PCR product		10.0
2.	Water		7.6
3.	Buffer (10X)	IX	2.0
4.	BSA(IOOX)	IX	0.2
5.	Enzyme (10 U/ul)	2 units	0.2

Total volume = 20 ul

RESTRICTION SITE OF ENZYMES

TaqI 5' ... TACGA... 3' 3' ... AGCAT... 5'

PROCEDURE:

The reaction mixture was prepared to a total volume of 20 ul. The samples were kept for digestion at 65°C in a water bath for 2 hours. 10 ul of the digested product was mixed with 2 ul of 6X gel loading dye and electrophoresed on a 2% agarose gel containing EtBr (0.5 ug/ml), along side a l00bp marker (Bangalore Genei).

STATISTICAL ANALYSIS

Statistical analysis was done using Statistical Package for the Social Sciences SPSS software.

HARDY WEINBERG PRINCIPLE / EQUILIBRIUM / LAW

The Hardy-Weinberg principle states that both allele and genotype frequencies in a population remain constant or are in equilibrium from generation to generation unless specific disturbing influences are introduced. These disturbing influences include non-random mating, mutations, selection, limited population size, random genetic drift and gene flow.

In the simplest case of a single locus with two alleles: the dominant allele is denoted A and the recessive a and their frequencies are denoted by p and q; freq(A)=p; freq(a)=#; p + q = 1. If the population is in equilibrium, then we will have freq(AA)=p for the AA homozygotes in the population, freq(aa)=^2 for the aa homozygotes, and freq(Aa)=2p# for the heterozygotes.

RESULTS

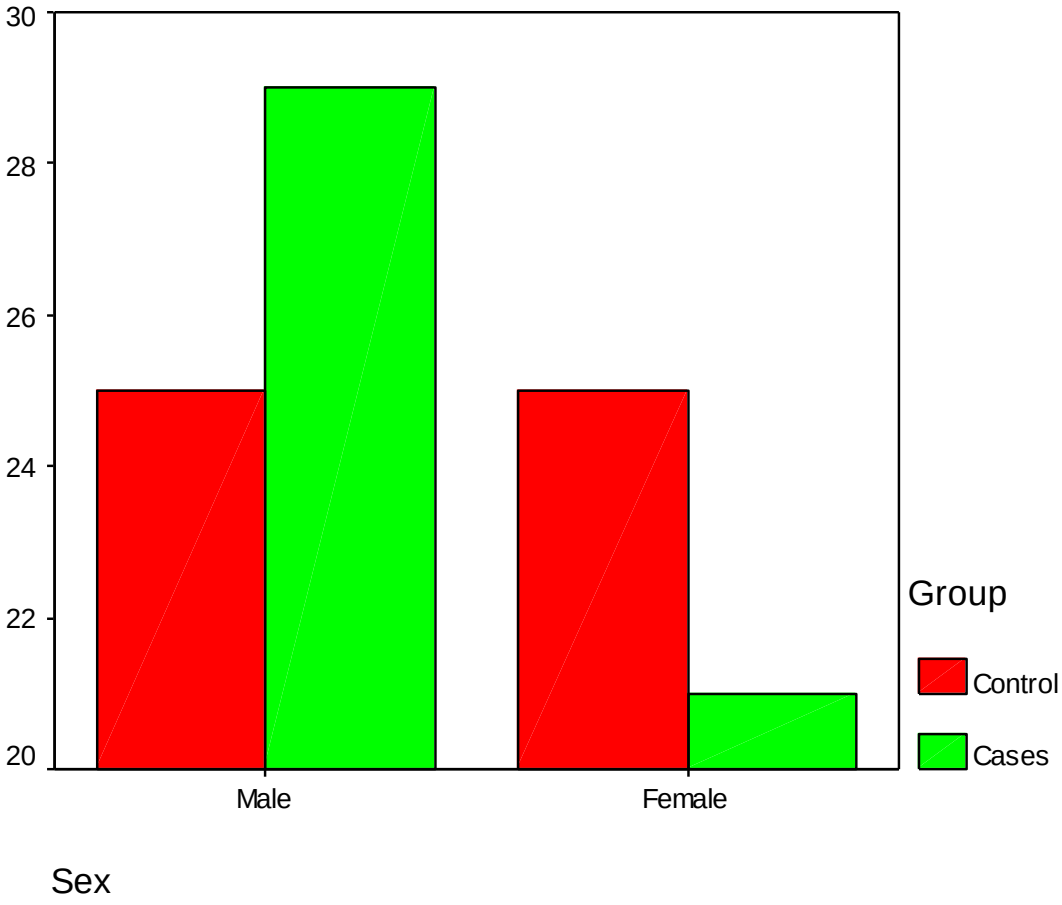
Calcium nephrolithiasis cases:

Among fifty stone disease patients who belonged to south indian population, 29 cases (53.7%) were males and 21 cases(45.7%) were females. The average age of cases was 41.68 with a standard deviation of 12.94, whereas the average age of the controls was 44.18 with a standard deviation of 12.81.There was no statistical significance between the age values.

Case distribution between sexes

			Group		***Total***	***P value***
			Control	***Cases***		
Sex	***Male***	Count	**25**	**29**	**54**	0.422
		% within Sex	46.3%	53.7%	100.0%	
		% within Group	50.0%	58.0%	54.0%	
	Female	Count	**25**	**21**	**46**	
		% within Sex	54.3%	45.7%	100.0%	
		% within Group	50.0%	42.0%	46.0%	
Total		Count	**50**	**50**	**100**	
		% within Sex	50.0%	50.0%	100.0%	
		% within Group	100.0%	100.0%	100.0%	

Though the number of male cases were more when compared to female cases ,this lot was chosen randomly from a huge pool of patients visiting Urology department, RGGGH and there was no statistically significant more number among the male group owing to the small sample size.



Mean age value of cases and controls

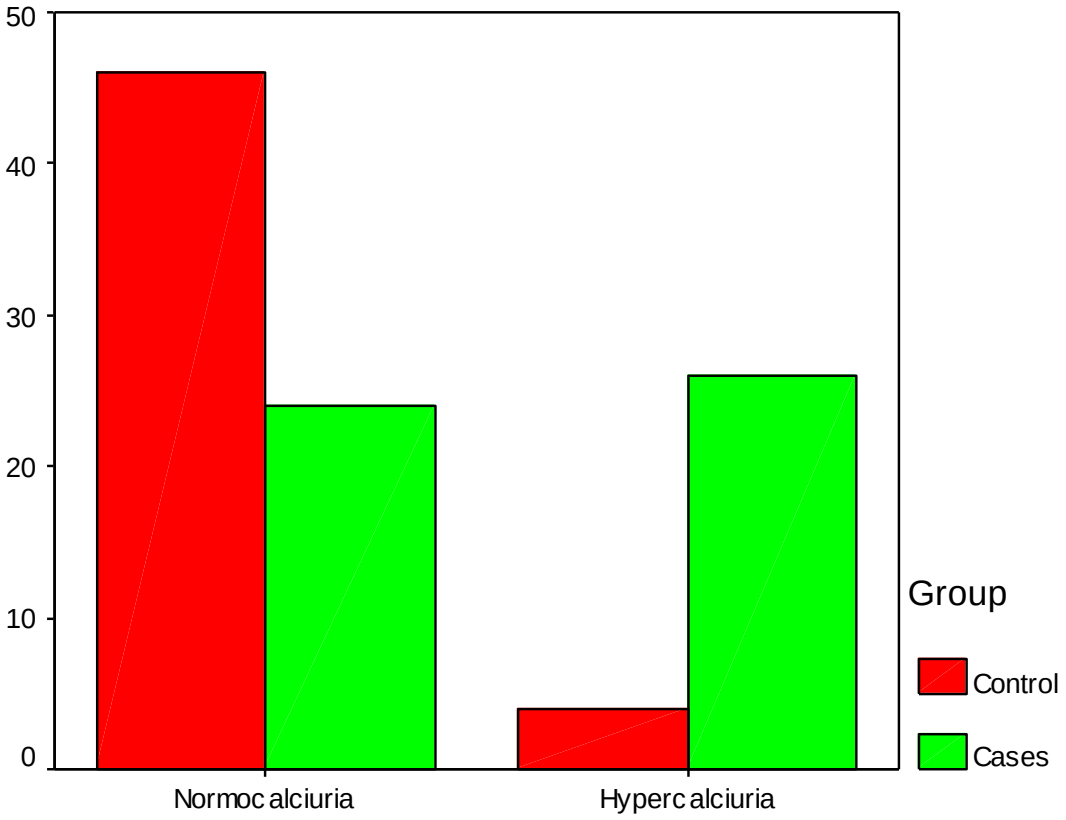
	Group	N	Mean	Std. Deviation	P value
Age in years	**Control**	50	44.18	12.814	0.334
	Cases	50	41.68	12.940	

ANALYSIS OF 24 HR URINARY CALCIUM VALUES:

There were 26 stone formers with elevated 24 hr urinary calcium levels accounting to 52% of stone disease patients.The reported incidence of hypercalciuria in Calcium nephrolithiasis is in the range of 33% to 52% according to various studies reported in literature. A point to be noted here is even 4 (8%)controls who never had any incidence or family history of stone disease also had hypercalciuria.

24 hr urinary calcium in cases and controls

			Group		*Total*	*P value*
			Control	**Cases**		
24 hr urinary calcium (mg/kg/day)	***Normocalciuria***	Count	**46**	**24**	**70**	***<0.001***
		% within 24 hr urinary calcium (mg/kg/day)	65.7%	34.3%	100.0%	
		% within Group	92.0%	48.0%	70.0%	
	Hypercalciuria	Count	**4**	**26**	**30**	
		% within 24 hr urinary calcium (mg/kg/day)	13.3%	86.7%	100.0%	
		% within Group	8.0%	52.0%	30.0%	***<0.001***
Total		Count	50	50	100	
		% within 24 hr urinary calcium (mg/kg/day)	50.0%	50.0%	100.0%	
		% within Group	100.0%	100.0%	100.0%	

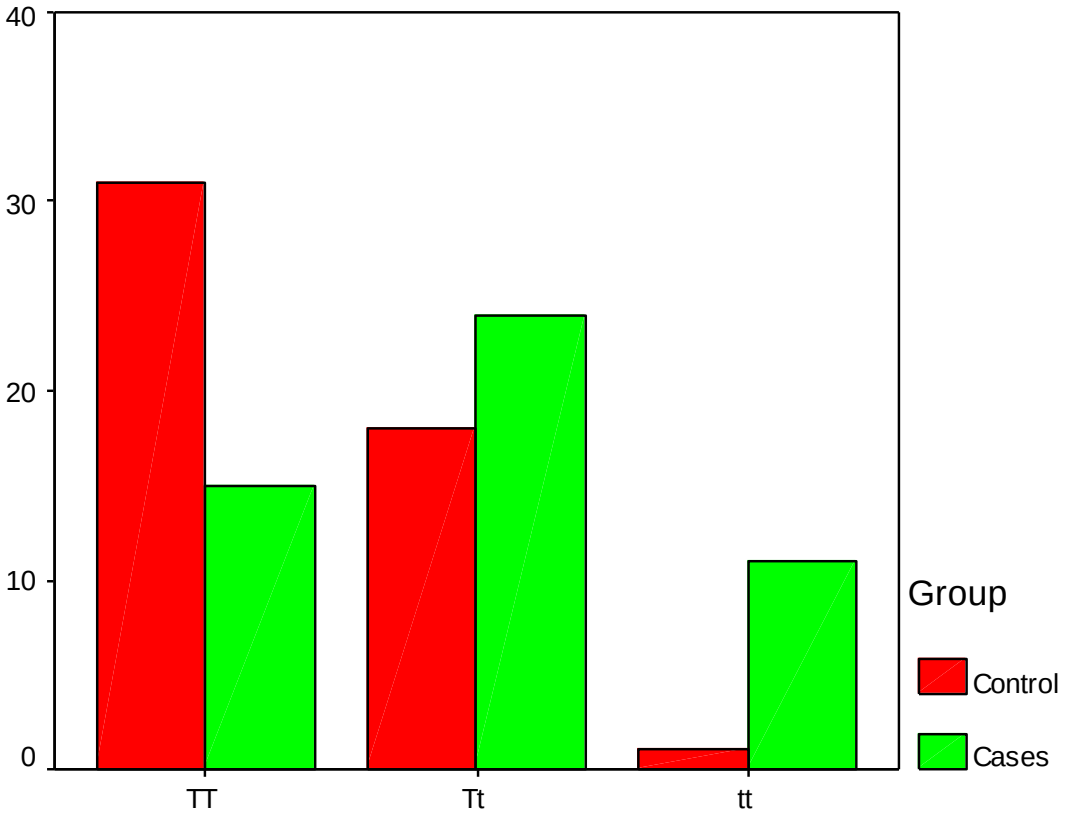


Hypercalciuria was statistically more significant among cases when compared to controls. But an interesting finding as proven by previous similar studies to be noted here is that hypercalciuria is not universal in all calcium stone diseases.This exposes the lacuna in knowledge that there are other genetic or metabolic causes which may play an additional role in calcium nephrolithiasis. This clearly explains the fact calcium nephrolithiasis is multifactorial and has so many other determinants to play a role.

Distribution of Taq I genotype between groups

			Group		*Total*	*P value*
			Control	***Cases***		
Taq 1 genotype	***TT***	Count	**31**	**15**	**46**	
		% within Taq 1 genotype	67.4%	32.6%	100.0%	**0.001**
		% within Group	62.0%	30.0%	46.0%	
	Tt	Count	**18**	**24**	**42**	
		% within Taq 1 genotype	42.9%	57.1%	100.0%	**<0.001**
		% within Group	36.0%	48.0%	42.0%	
	tt	Count	**1**	**11**	**12**	
		% within Taq 1 genotype	8.3%	91.7%	100.0%	**<0.001**
		% within Group	2.0%	22.0%	12.0%	
Total		Count	**50**	**50**	**100**	
		% within Taq 1 genotype	50.0%	50.0%	100.0%	
		% within Group	100.0%	100.0%	100.0%	

Among the various polymorphic genotypes the presence of "t" allele strongly has a positive impact on stone formation.The number of tt polymorphs with stone disease was 11(ie. 91.7% of total tt population) .This clearly proves the statistically significant increased risk of stone formation in the presence of tt genotype.In the Tt allelic group 24 (57.1%) were stone formers having statistically significant increased chance of stone formation proving the role of "t" allele linked to stone disease .Whereas in TT allelic group most of them were controls ,31in number forming 62% of control limb .



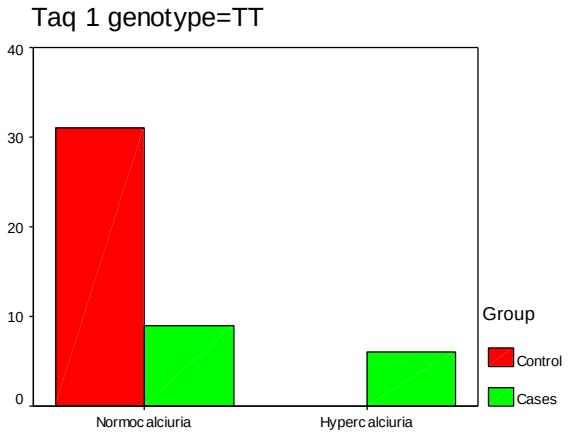
Comparing 24 hr urinary calcium among various Taq 1 genotypes:

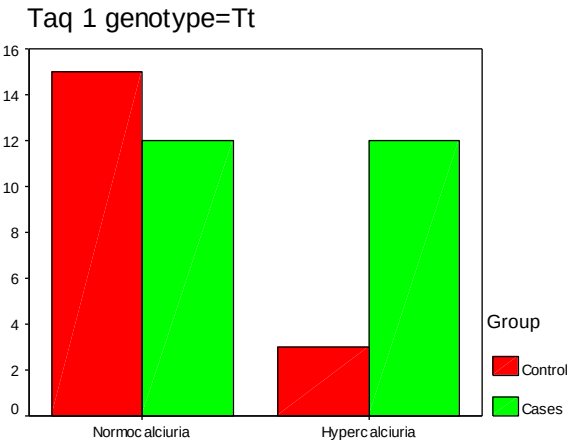
Among 11 cases of tt polymorphs 9 had hypercalciuria(8 cases and one control) forming 75% of the group. Hypercalciuria was statistically more significant in tt polymorphs. A similar observation was seen with Tt cases thereby re-emphasizing the role of "t" allele in hypercalciuria and nephrolithiasis.

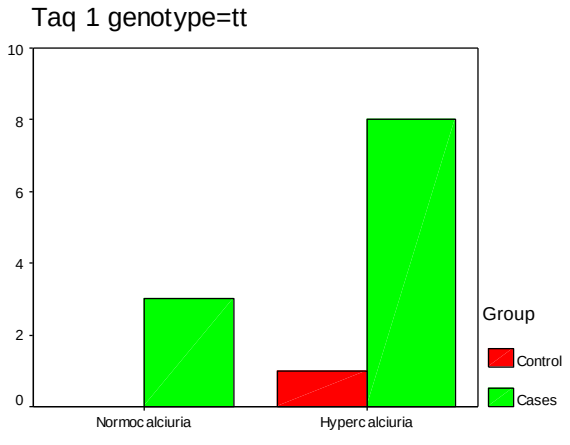
Comparing 24 hr urinary calcium among various Taq 1 genotypes:

Taq 1 genotypes				*Group*		*Total*	*P value*
				Control	*Cases*		
TT	***24 hr urinary calcium (mg/kg/day)***	***Normocalciuria***	Count	**31**	**9**	**40**	
			% within 24 hr urinary calcium (mg/kg/day)	77.5%	22.5%	100.0%	
			% within Group	100.0%	60.0%	87.0%	
		Hypercalciuria	Count	**0**	**6**	**6**	**0.001**
			% within 24 hr urinary calcium (mg/kg/day)	.0%	100.0%	100.0%	
			% within Group	.0%	40.0%	13.0%	
	Total		Count	**31**	**15**	**46**	
			% within 24 hr urinary calcium (mg/kg/day)	67.4%	32.6%	100.0%	
			% within Group	100.0%	100.0%	100.0%	
Tt	***24 hr urinary calcium (mg/kg/day)***	***Normocalciuria***	Count	**15**	**12**	**27**	
			% within 24 hr urinary calcium (mg/kg/day)	55.6%	44.4%	100.0%	
			% within Group	83.3%	50.0%	64.3%	**0.027**
		Hypercalciuria	Count	3	12	15	
			% within 24 hr urinary calcium (mg/kg/day)	20.0%	80.0%	100.0%	
			% within Group	16.7%	50.0%	35.7%	
	Total		Count	**18**	**24**	**42**	
			% within 24 hr urinary calcium (mg/kg/day)	42.9%	57.1%	100.0%	
			% within Group	100.0%	100.0%	100.0%	
tt	***24 hr urinary calcium (mg/kg/day)***	***Normocalciuria***	Count	**0**	**3**	**3**	
			% within 24 hr urinary calcium (mg/kg/day)	.0%	100.0%	100.0%	
			% within Group	.0%	27.3%	25.0%	
		Hypercalciuria	Count	**1**	**8**	**9**	
			% within 24 hr urinary calcium (mg/kg/day)	11.1%	88.9%	100.0%	**0.040**
			% within Group	100.0%	72.7%	75.0%	
	Total		Count	**1**	**11**	**12**	
			% within 24 hr urinary calcium (mg/kg/day)	8.3%	91.7%	100.0%	
			% within Group	100.0%	100.0%	100.0%	

Comparing 24 hr urinary calcium among various Taq 1 genotypes:



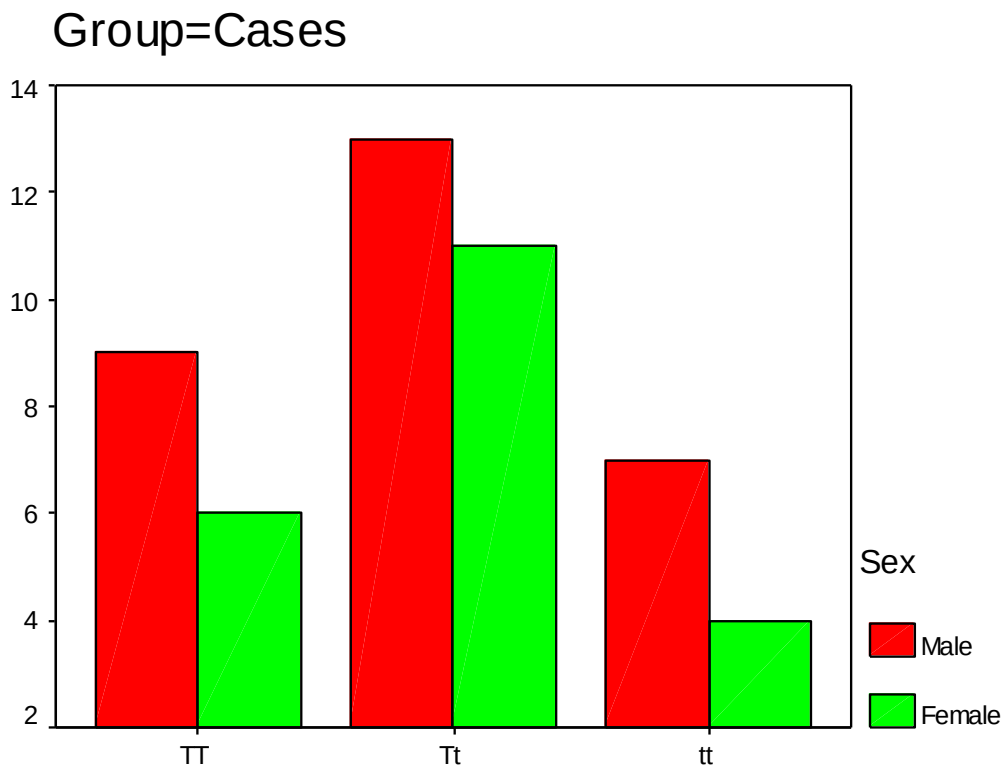




Analysis of Taq I genotype between the sexes

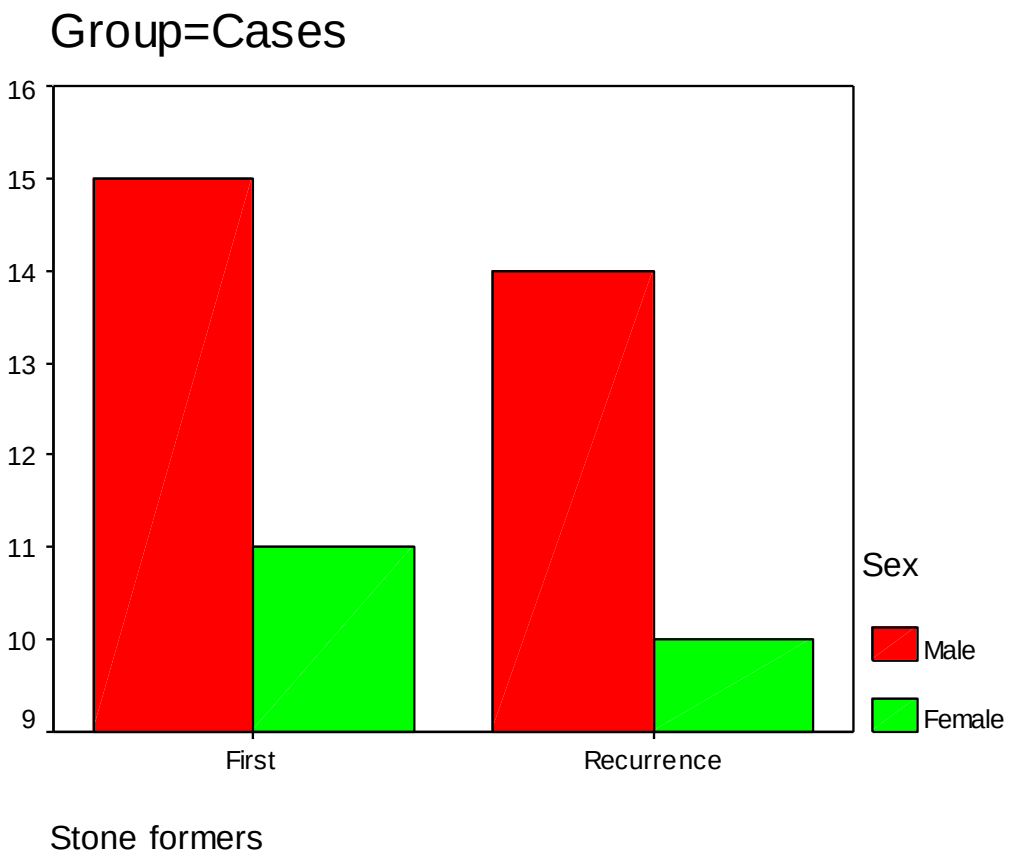
Group				Sex		Total	P value
				Male	Female		
Control	**Taq 1 genotype**	**TT**	Count	**14**	**17**	**31**	0.469
			% within Taq 1 genotype	45.2%	54.8%	100.0%	
			% within Sex	56.0%	68.0%	62.0%	
		Tt	Count	**10**	**8**	**18**	0.387
			% within Taq 1 genotype	55.6%	44.4%	100.0%	
			% within Sex	40.0%	32.0%	36.0%	
		tt	Count	**1**	**0**	**1**	0.290
			% within Taq 1 genotype	100.0%	.0%	100.0%	
			% within Sex	4.0%	.0%	2.0%	
	Total		Count	25	25	50	
			% within Taq 1 genotype	50.0%	50.0%	100.0%	
			% within Sex	100.0%	100.0%	100.0%	
Cases	**Taq 1 genotype**	**TT**	Count	**9**	**6**	**15**	0.855
			% within Taq 1 genotype	60.0%	40.0%	100.0%	
			% within Sex	31.0%	28.6%	30.0%	
		Tt	Count	**13**	**11**	**24**	0.855
			% within Taq 1 genotype	54.2%	45.8%	100.0%	
			% within Sex	44.8%	52.4%	48.0%	
		tt	Count	**7**	**4**	**11**	0.899
			% within Taq 1 genotype	63.6%	36.4%	100.0%	
			% within Sex	24.1%	19.0%	22.0%	
	Total		Count	**29**	**21**	**50**	
			% within Taq 1 genotype	58.0%	42.0%	100.0%	
			% within Sex	100.0%	100.0%	100.0%	

There was no statistically significant difference in the number of male and female cases and controls when comparing the incidence of various Taq I gene polymorphs. No sex predilection of a particular type of polymorph was noticed.



Comparing recurrent stone formers with sex

Group				Sex		Total	P value
				Male	Female		
Cases	Stone formers	First	Count	**15**	**11**	**26**	
			% within Stone formers	57.7%	42.3%	100.0%	**0.963**
			% within Sex	51.7%	52.4%	52.0%	
		Recurrence	Count	**14**	**10**	**24**	
			% within Stone formers	58.3%	41.7%	100.0%	**0.963**
			% within Sex	48.3%	47.6%	48.0%	
	Total		Count	**29**	**21**	**50**	
			% within Stone formers	58.0%	42.0%	100.0%	
			% within Sex	100.0%	100.0%	100.0%	



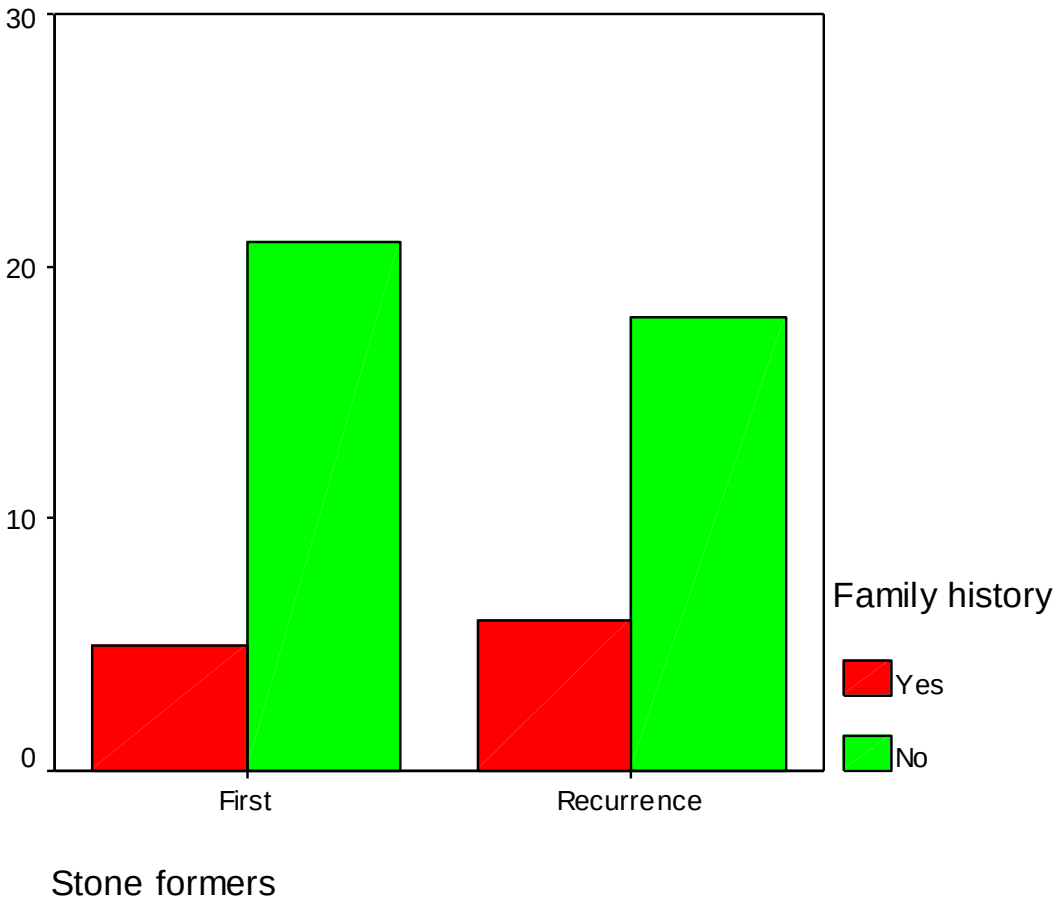
Though the number of male stone formers with recurrent disease appears to be more(14/24) there was no statistically significant sex predilection towards recurrent stone formers.

Comparing the family history of stone formers:

Family history was positive in 6 recurrent stone formers and 5 first time stone formers depicting the absence of any statistically significant association between family history and recurrence in our study. It's unusual to see that the family history was positive in only 22% of stone forming cases(11 out of 50 cases).

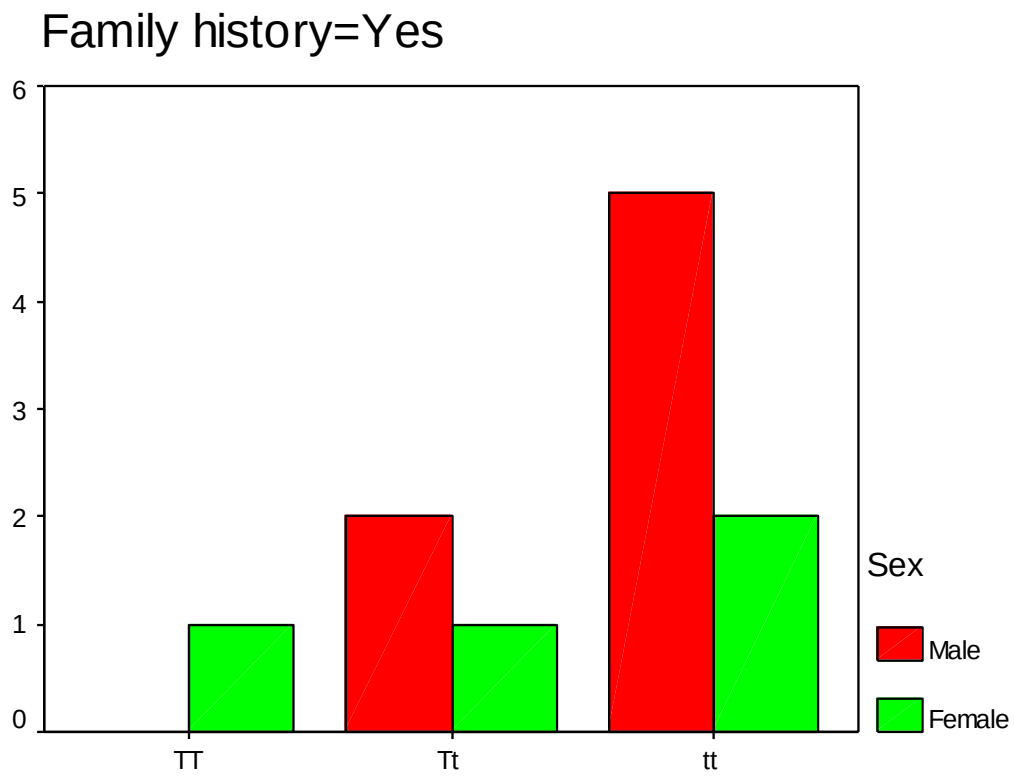
Comparing the family history of stone formers:

			Family history		Total	P value
			Yes	No		
Stone formers	**First**	Count	**5**	**21**	**26**	
		% within Stone formers	19.2%	80.8%	100.0%	**0.623**
		% within Family history	45.5%	53.8%	52.0%	
	Recurrence	Count	**6**	**18**	**24**	
		% within Stone formers	25.0%	75.0%	100.0%	**0.821**
		% within Family history	54.5%	46.2%	48.0%	
Total		Count	**11**	**39**	**50**	
		% within Stone formers	22.0%	78.0%	100.0%	
		% within Family history	100.0%	100.0%	100.0%	



Correlating Taq I genotypes with family history:

Family history				Sex		Total	P value
				Male	Female		
Yes	Taq 1 genotype	TT	Count	0	1	1	0.370
			% within Taq 1 genotype	.0%	100.0%	100.0%	
			% within Sex	.0%	25.0%	9.1%	
		Tt	Count	2	1	3	0.329
			% within Taq 1 genotype	66.7%	33.3%	100.0%	
			% within Sex	28.6%	25.0%	27.3%	
		tt	Count	5	2	7	0.281
			% within Taq 1 genotype	71.4%	28.6%	100.0%	
			% within Sex	71.4%	50.0%	63.6%	
	Total		Count	7	4	11	
			% within Taq 1 genotype	63.6%	36.4%	100.0%	
			% within Sex	100.0%	100.0%	100.0%	
No	Taq 1 genotype	TT	Count	9	5	14	0.756
			% within Taq 1 genotype	64.3%	35.7%	100.0%	
			% within Sex	40.9%	29.4%	35.9%	
		Tt	Count	11	10	21	0.754
			% within Taq 1 genotype	52.4%	47.6%	100.0%	
			% within Sex	50.0%	58.8%	53.8%	
		tt	Count	2	2	4	0.491
			% within Taq 1 genotype	50.0%	50.0%	100.0%	
			% within Sex	9.1%	11.8%	10.3%	
	Total		Count	22	17	39	
			% within Taq 1 genotype	56.4%	43.6%	100.0%	
			% within Sex	100.0%	100.0%	100.0%	



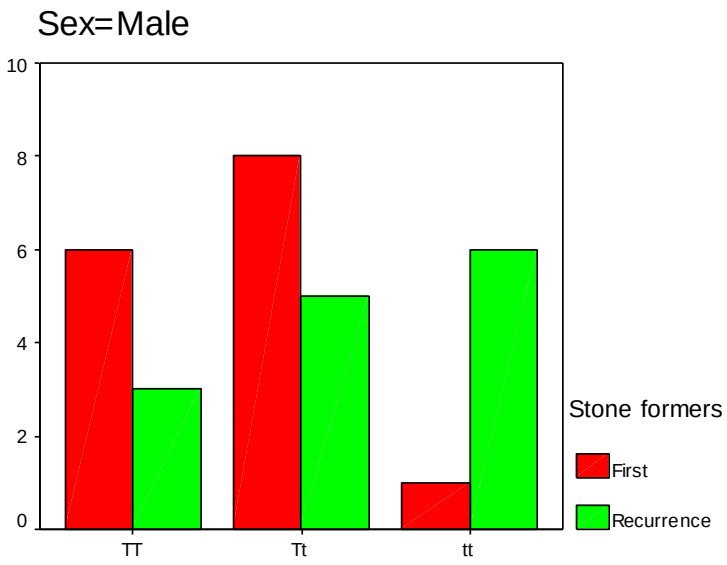
Family history was not statistically significant between the three types of polymorphs. Numerically tt polymorphs had the highest number of positive family history(7 out of 11 cases) contributing 63.6 % to the group. Though this value appears to be high, pointing a correlation between tt genotype and family history , is not statistically significant.

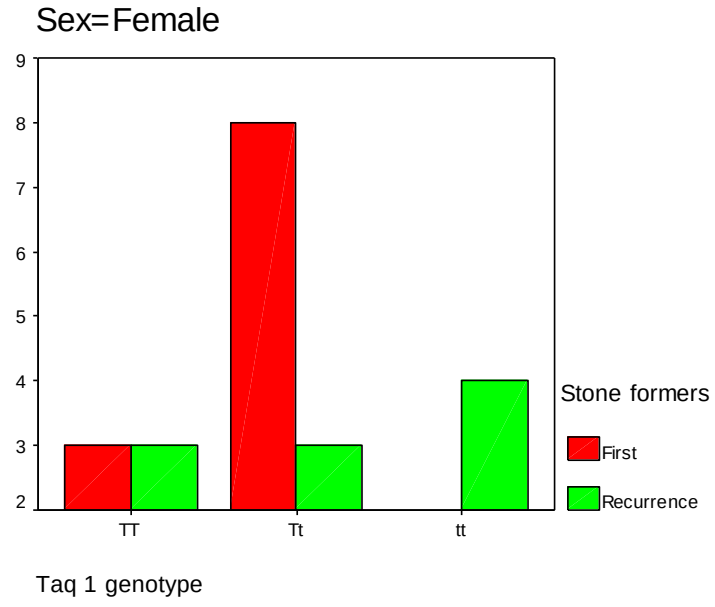
Most of family history positive cases are males belonging to both tt and Tt genotypes once again giving an idea that the “t” allele may have a positive role in family history.

Comparing Taq 1 genotypes with stone formers:

Sex				Stone formers		Total	P value
				First	Recurrence		
Male	**Taq 1 genotype**	**TT**	Count	6	3	9	0.073
			% within Taq 1 genotype	66.7%	33.3%	100.0%	
			% within Stone formers	40.0%	21.4%	31.0%	
		Tt	Count	8	5	13	0.059
			% within Taq 1 genotype	61.5%	38.5%	100.0%	
			% within Stone formers	53.3%	35.7%	44.8%	
		tt	Count	**1**	**6**	**7**	**0.049**
			% within Taq 1 genotype	14.3%	85.7%	100.0%	
			% within Stone formers	6.7%	42.9%	24.1%	
	Total		Count	**15**	**14**	**29**	
			% within Taq 1 genotype	51.7%	48.3%	100.0%	
			% within Stone formers	100.0%	100.0%	100.0%	
Female	**Taq 1 genotype**	**TT**	Count	**3**	**3**	**6**	**0.044**
			% within Taq 1 genotype	50.0%	50.0%	100.0%	
			% within Stone formers	27.3%	30.0%	28.6%	
		Tt	Count	**8**	**3**	**11**	**0.023**
			% within Taq 1 genotype	72.7%	27.3%	100.0%	
			% within Stone formers	72.7%	30.0%	52.4%	
		tt	Count	**0**	**4**	**4**	**0.022**
			% within Taq 1 genotype	.0%	100.0%	100.0%	
			% within Stone formers	.0%	40.0%	19.0%	
	Total		Count	**11**	**10**	**21**	
			% within Taq 1 genotype	52.4%	47.6%	100.0%	
			% within Stone formers	100.0%	100.0%	100.0%	

Stratifying Taq I genotype among male stone formers:





The recurrence of stone disease among male population was high in tt genotype, with definitive statistically significant increase.Among female cases recurrence of stone disease was statistically significant in Tt and tt polymorphs. Altogether tt genotype is responsible for recurrence of stone formation.

1. 91.7 % of tt genotypic individuals are stone formers.

2. Presence of "t" allele increases the risk for stone formation statistically.

3. The percentage of stone disease is least in TT genotype.

4. Hypercalciuria was seen in 75% of tt genotypes. Both 'tt' and 'Tt' genotypes have a statistically significant increase in the incidence of hypercalciuria.

5. No sex predilection was noticed for a particular phenotype.

6. Though the number of male stone formers with recurrent disease are more(14/24) there is no statistical significance to it.

7. Family history was positive in only 22 % of stone formers and no specific genotype was linked to this entity.

8. Numerically’ tt’ polymorphs had the highest number of positive family history(7 out of 11 cases) contributing 63.6 % to the group but the value is not statistically significant.

9. ‘tt’ males had an increased chance of stone recurrence whereas Tt and tt females had an increased recurrence chance ,both proven statistically significant.

DISCUSSION

The present study investigated polymorphism in Vitamin D receptor gene Taq I and the risk of calcium nephrolithiasis in South Indian population. There are no studies from South India on this research till date.There are studies published from North India and these studies invoived only one Vitamin D receptor polymorphism Fok-I(H.K.Bid et al). Thus this the only Indian study looking for the role of Taq I gene polymorphism in calcium urolithiasis.

The results of present study were compared with a systemic meta analysis

Year	Reference (first author)	Region	Stone composition[1]	Cases	Controls	Polymorphic sites (HWE status[2])
1999	Ruggiero [20]	Europe	NS	27	150	*Bsm*I (N)
1999	Jackman [7]	America	Calcium	17	37	*Taq*I (N)
2001	Chen [10]	Asia	Calcium	124	90	*Bsm*I (N)
2001	Chen [21]	Asia	Calcium	146	90	*Fok*I (Y)
2002	Nishijima [23]	Asia	Calcium	83	83	*Apa*I (Y), *Taq*I (Y)
2003	Ozkaya [25]	Europe	Calcium	64	90	*Apa*I (N), *Bsm*I (Y), *Taq*I (Y)
2003	Wang [34]	Asia	Calcium	150	80	*Apa*I (Y), *Fok*I (Y), *Taq*I (Y)
2004	Mossetti [32]	Europe	Calcium	110	127	*Taq*I (Y)
2004	Rendina [27]	Europe	Calcium	159	124	*Apa*I (Y), *Bsm*I (Y), *Fok*I (Y)
2004	Relan [26]	Asia	Calcium	150	100	*Bsm*I (N), *Fok*I (N)
2005	Bid [28]	Asia	Calcium	50	60	*Fok*I (N)
2005	Bid [33]	Asia	Calcium	138	166	*Fok*I (N)
2006	Gunes [12]	Europe	Calcium	110	150	*Apa*I (Y), *Bsm*I (Y), *Taq*I (Y)
2007	Liu [29]	Asia	Calcium	235	231	*Fok*I (Y)
2007	Seyhan [31]	Europe	Calcium	80	40	*Taq*I (N)
2009	Seo [11]	Asia	NS	278	535	*Apa*I (N), *Fok*I (N), *Taq*I (N)
2010	Mittal [9]	Asia	NS	125	150	*Apa*I (Y), *Fok*I (N), *Taq*I (Y)

(1) 'NS' – Unspecified stone composition , while 'Calcium' indicates calcium stone. 'N' indicates the genotype distribution of the corresponding control group deviated from HWE, 'Y' indicates the consistence with HWE. (2) HWE. Hardy Weinburgh Equilibirium)

Calcium mineral metabolism is altered by altered vitamin D receptor gene polymorphisms. There are so many studies to prove the association of this gene with osteoporosis, prostatic carcinoma, Psoriasis and urolithiasis. Basically the polymorphism in the untranslated region of VDR gene ie. TaqI, BsmI and ApaI don't actually change the amino acid sequence. Anyhow the hypothesized mechanism of stone formation is due to alteration in mRNA stability or a change in vitamin D activity.But these presumptions are not scientifically proven till date.

There are various studies conducted world over for and against VDR polymorphism in gene formation. Nothing is conclusive among published results and no association of genetic polymorphism is actually supported.

In previous studies, Jackman *et al*. in 1999 showed the association between 'TT' genotype and stone formation in western population. This is completely contrary to what we have noticed in our study. In our study 'TT' genotype has the least risk of stone formation .

But the study done by Nishijima et al in a group of Japanese Asian population showed a clear link between 'tt' genotype and stone formation.

The work done by Serkan Seyhan et al in Turkish children has results similar to our study which links the presence of 't' allele to recurrent stone formation. But this Turkish study links the presence of 't' allele to positive

family history which was not observed in our study. The educational status and intelligent quotient of the patient including his socioeconomic back ground has a strong influence in getting a proper history from a patient. That may be the reason why the percentage of cases with positive family history is too low in our setup owing to many of family history being missed because of patient's unawareness.This Turkish study also had a significant correlation with hypercalciuria especially in cases with Tt,tt genotypes which is similar to our report.

In other studies especially the one by Mosetti et al, they took hypocitraturia as a parameter and found that genetics polymorphs had a correlation with this parameter especially in recurrent stone disease. We didn't take urinary citrate as one of our parameters.

Ozkaya et al tested for polymorphism in other regions of VDR gene like Apa I.Bsm I including Taq I and found that AA polymorphs of Apa I had significantly more hypercalciuric urolithiasis.In contrary to our observation he found that there is no association with Taq I polymorphism and urolithiasis.

They also showed that the '*TT'* genotype is significantly associated with a positive family history.

Another study by, Gunes *et al*. found no correlation between stone risk and the three mentioned polymorphisms. The only finding noticed was Apa I polymorphism and positive family history.

The reason for such gross alterations in results was due to other factors like environmental factors, ethnicity and due to technical differences in different laboratories.

The meta analysis done by Yiwei Lin et al tabulates the results as follows

Category	OR (95% CI)	$P_{heterogeneity}$	I^2, %
Allelic comparison (t vs. T)			
All	1.15 (1.00, 1.34)	0.869	0
Asia	1.23 (0.99, 1.52)	0.567	0
Europe	1.09 (0.88, 1.35)	0.754	0
America	–	–	–
Calcium stone	1.16 (0.98, 1.38)	0.718	0
All in HWE	1.15 (0.98, 1.36)	0.674	0
Dominant model (Tt + tt vs. TT)			
All	1.28 (1.05, 1.56)	0.811	0
Asia	1.40 (1.07, 1.82)	0.621	0
Europe	1.11 (0.81, 1.52)	0.7	0
America	–	–	–
Calcium stone	1.26 (0.98, 1.61)	0.758	0
All in HWE	1.34 (1.07, 1.69)	0.656	0
Recessive model (tt vs. TT + Tt)			
All	1.04 (0.77, 1.40)	0.254	21.3
Asia	0.95 (0.58, 1.56)	0.35	8.5
Europe	1.15 (0.78, 1.71)	0.101	51.9
America	–	–	–
Calcium stone	1.15 (0.83, 1.60)	0.192	31
All in HWE	0.96 (0.69, 1.33)	0.464	0

The Odd's ratio within 95% confidence intervals is found to be slightly more in both in allelic comparisons and Dominant models. It's interesting to

note that the Odd's Ratio value is also slightly higher for Asian population just like our study, clearly stating that ethnicity has an important role in polymorphism and it's effects.

The VDR gene regulates the following factors apart from Vitamin D: osteocalcine, parathyroid hormone, calcitonine and various oncoproteins . As there are so many studies which correlate VDR gene polymorphism and prostatic cancer, parallel association studies between calcium urolithiasis and carcinoma prostate are almost not known in the literature.

VDR polymorphisms alters the mRNA transcription thereby being quoted in various other disorders.In a study the 'T' allele was found to decrease the chance of osteoporosis but paradoxically was associated with increased chance of osteoarthritis. Another research paper on idiopathic hypercalciuria in children has observed idiopathic hypercalciuria to be found in association with severe osteopenia in the study group children.Idiopathic hypercalciuria-osteoporosis-calcium urolithiasis all are linked in several ways.

In conclusion, pathophysiology of urinary stone disease includes several previously described components, such as gender, ethnicity, environmental and dietary factors. Genetics is the only key to open this pandora's box. As prevention is better than cure these genetic studies aiming at detecting genetic markers are much more important. In future these studies will help human

community in prophylaxis against stone formation and identifying high risk individuals in preventing the recurrence of disease problem.

However, this study is just a beginning of a long journey and further studies planned in large scale with huge sample with various other risk factors comparing different races is required in the future. Our study is just a piece of evidence in analyzing the genetics of calcium stone disease, thereby forming a block of an unsolved puzzle.

CONCLUSIONS

- The presence of “t” allele increases the risk for stone formation statistically.
- Both tt and Tt genotypes have a statistically significant increase in the incidence of hypercalciuria.
- No specific genotype has a statistically significant association with a positive family history.
- ‘tt’ males had an increased chance of stone recurrence whereas ‘Tt’ and ‘tt’ females had an increased recurrence statistically.

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INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No : 044 25305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To
Dr. S. Ganesh Prasad
PG in MCH Urology
Madras Medical College, Chennai -3

Dear Dr. S. Ganesh Prasad

The Institutional Ethics committee of Madras Medical College, reviewed and discussed your application for approval of the proposal entitled "Role of vitamin D receptor gene Taq I polymorphism in recurrent urolithiasis " No.25062012.

The following members of Ethics Committee were present in the meeting held on 19.06.2012 conducted at Madras Medical College, Chennai -3.

1.	Dr. S.K. Rajan. M.D.,FRCP.,DSc	-- Chairperson
2.	Prof. K. Ramadevi MD Prof of Biochemistry, MMC, Ch-3	-- Member
3.	Prof. R. Nandhini MD Director, Inst. of Pharmacology ,MMC, Ch-3	-- Member
4.	Prof. C. Rajendiran, MD Director , Inst. of Internal Medicine, MMC, Ch-3	-- Member
5.	Prof. S. Deivanayagam MS Prof of Surgery, MMC, Ch-3	-- Member
6.	Prof. A. Radhakrishnan MD Prof of Internal Medicine, MMC, Ch-3	-- Member

We approve the proposal to be conducted in its presented form.

Sd/ Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, and SAE occurring in the course of the study, any changes in the protocol and patients information /.informed consent and asks to be provided a copy of the final report.

Member Secretary, Ethics Committee

CONSENT FORM

STUDY TITLE : "ROLE OF VITAMIN D RECEPTOR GENE TAQ I POLYMORPHISM IN RECURRENT UROLITHIASIS"

I _____ hereby give consent to participate in the study conducted by DR. S.GANESH PRASAD, 2nd Year MCh Urology Postgraduate student , Madras Medical College and Government General Hospital, Chennai – 3, and to use my personal clinical data and result of investigation for the purpose of analysis. I also give consent for withdrawing and using my blood and urine sample for genetic , hormonal analysis and further investigations.

Signature / Thumb impression of the patient / relative

Place

Date

Patient Name and Address

Signature of the Investigator

ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சி தலைப்பு : திரும்பத் திரும்ப வரும் சிறுநீரகக் கற்களும், வைட்டமின்-டி ஏற்பி மரபணு பல உருவியலும் பற்றிய ஆய்வு.

பெயர் : தேதி :

வயது : உள் நோயாளி எண் :

பால் : ஆராய்ச்சி சேர்க்கை எண் :

இந்த ஆராய்ச்சியின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது.

எனக்கு விளக்கப்பட்ட விஷயங்களை நான் புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

எனது உதிரத்திலிருந்து வைட்டமின்-டி ஏற்பியின் மரபணு சம்பந்தமான பரிசோதனையும், சிறுநீரிலிருக்கும் கால்சியம் அளவு கண்டுபிடிக்கும் பரிசோதனையும் செய்துகொள்ள சம்மதிக்கிறேன்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்ப்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரின் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும் அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் நான் புரிந்து கொண்டேன்.

நான் சிறுநீரகக் கற்களுக்கான மரபணு காரணம் குறித்த இந்த ஆராய்ச்சியின் விவரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக் கொண்டேன்.

நான் என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக் கொள்ள சம்மதிக்கிறேன்.

கையொப்பம்.

INFORMATION SHEET:

Your blood and urine specimen has been accepted.

We are conducting a study on genetic analysis of stone disease involving kidney among patients attending Government General Hospital, Chennai and for that your specimen may be valuable to us.

The purpose of this study is to predict recurrent kidney stone formers with the help of certain special tests.

We are selecting certain cases and if your specimen is found eligible, we may be using your specimen to perform extra tests and special studies which in any way do not affect your final report or management.

The privacy of the patients in the research will be maintained throughout the study .In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.

The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of investigator Signature of the patient

Date:

ஆராய்ச்சி தகவல் தாள்

தங்களது உதிரமும், சிறுநீரும் பரிசோதனைக்காக இங்கு பெறப்பட்டுள்ளது.

சென்னை ராஜீவ் காந்தி அரசு பொது மருத்துவமனைக்கு வரும் நோயாளிகளிடம் இருக்கும் சிறுநீரகக் கற்கள் உருவாகக் காரணமான வைட்டமின் டி ஏற்பியின் மரபணு பற்றிய ஒரு ஆராய்ச்சி இங்கு நடைபெற்று வருகின்றது.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். இந்த ஆராய்ச்சியில் தங்களது உதிரம் மற்றும் சிறுநீரை சிற்சில பரிசோதனைக்கு உட்படுத்துவோம். அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு ஏற்படாது என்பதையும் தெரிவித்துக்கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிட மாட்டோம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது. மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக்கொள்ளலாம்.

இந்த சிறப்புப் பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி:

PROFORMA

ROLE OF VITAMIN D RECEPTOR GENE Taq 1 POLYMORPHISM IN RECURRENT UROLITHASIS

Patient's name : .....

Age/sex : .....

IP NO: : .....

Contact address : .....

.....

.....

Contact number : .....

Family History of Stone disease : .....

Type of Stone : .....

First time (or) recurrent stone former : .....

24 Hr. Urinary Calcium : .....

Serum Calcium : .....

Serum Phosphorus : .....

Serum 1,25 $(OH)_2$ D_3 Level : .....

Taq 1 Genotype : .....

MASTER CHART – CASES

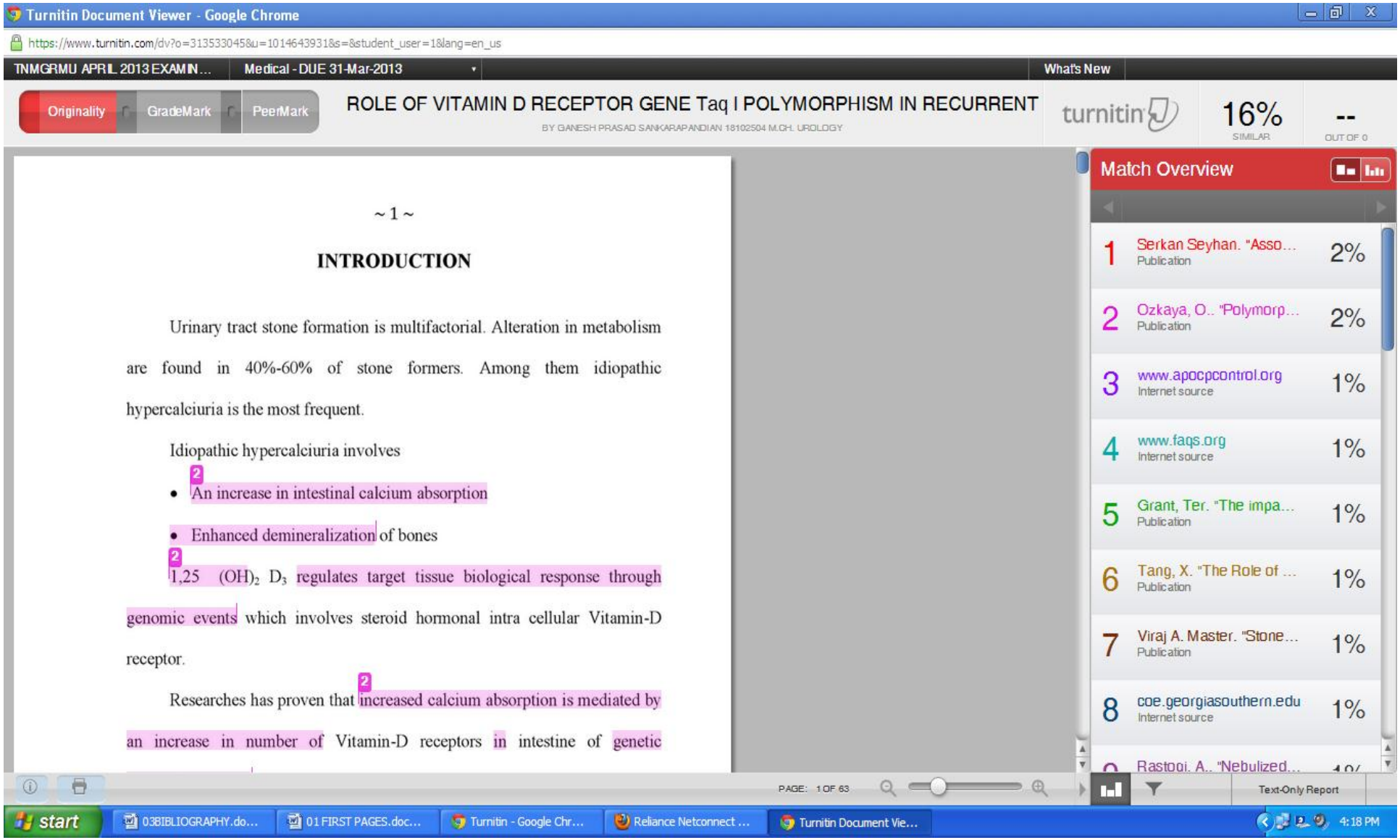
NAME	IP NO	AGE	SEX	FAMILY HISTORY	FIRST TIME/ RECURRENT	24 Hr URINARY CALCIUM (mg/Kg/day)	SERUM CALCIUM (mg/dl)	SERUM PHOSPHORUS (mg/dl)	SERUM VITAMIN D3(pg/ml)	Taq 1 GENOTYPE
Shankar	118696	42	Male	YES	F	6.3	10.6	4.3	28	Tt
Sekar	118676	35	Male	YES	F	6.1	9.8	5.1	29.4	Tt
Das	119923	35	Male	YES	R	6	10.6	4.8	30.2	tt
Vasantha	120813	40	Female	No	F	7.1	9.6	4.9	33.3	Tt
Faizal	119074	38	Male	No	F	6.4	10	5.2	34.1	TT
Sundar	118921	26	Male	No	R	6.8	9.7	6	35.3	Tt
Lakshmi	110656	50	Female	YES	F	6.2	10.2	4.9	32.2	Tt
Kumarasamy	115300	55	Male	No	F	6.2	10.1	4.2	33.2	TT
Sakthivel	117592	29	Male	No	R	5.8	9.8	4.6	33.5	Tt
Karuppayee	118811	47	Female	No	R	6	10.4	5.4	31.6	tt
Valliammal	113954	65	Female	No	R	4.8	9.7	6	36.4	TT
Sambandan	113956	59	Male	No	F	5.2	9.3	4.1	31.3	TT
Krishnaveni	113846	42	Female	No	R	6.2	10.1	5.2	34.4	tt
Patchiammal	110696	40	Female	No	F	6.4	10.2	4.6	36.6	Tt
Dhinesh	113994	28	Male	YES	R	6.8	9.1	5.3	39.4	tt
Nalini	112700	26	Female	YES	R	5.8	9.7	4.4	27.3	tt
Murugan	110119	34	Male	No	F	5.2	9.4	5.1	27.4	Tt
Soosai Rathnam	111490	39	Male	No	R	6.2	9.6	4.5	28.1	Tt
Amsa	111040	40	Female	No	F	6	9.8	5.3	27.2	Tt
Manikandan	111539	17	Male	No	F	3.8	9.4	5.1	27.3	TT
Kiruba rani	107585	30	Female	No	F	6.2	9.4	4.6	28.4	Tt
Arockiasamy	100272	51	Male	YES	F	7.2	9.6	4.9	29.1	tt
Soundarrajan	102907	55	Male	No	R	3	10.2	4.8	27.1	Tt
Lakshmi	102261	46	Female	YES	R	3.6	9.8	4.7	28.5	tt
Periyasamy	101405	49	Male	No	F	3.2	9.7	4.5	27.6	Tt

NAME	IP NO	AGE	SEX	FAMILY HISTORY	FIRST TIME/ RECURRENT	24 Hr URINARY CALCIUM (mg/Kg/day)	SERUM CALCIUM (mg/dl)	SERUM PHOSPHORUS (mg/dl)	SERUM VITAMIN D3(pg/ml)	Taq 1 GENOTYPE
Krishnammal	109821	34	Female	YES	F	4.8	9.4	4.4	27.8	TT
Rajavel	93995	54	Male	YES	R	5	9.2	4.6	27.9	tt
Sampoorna	97120	40	Female	No	F	3.6	9.4	5.3	27.4	TT
Manikandan	99058	28	Male	No	R	6.2	9.2	5.2	27.6	TT
Hambunisha	97160	28	Female	No	F	6	9.4	5.6	29.6	Tt
Ponnusamy	117187	64	Male	No	F	3.4	10.1	4.1	34.3	Tt
Mahendran	117215	45	Male	No	R	3.6	10.4	5.4	28.5	TT
Veerammal	119616	60	Female	No	F	3.2	9.5	5.7	33.3	Tt
Vijayalakshmi	120651	37	Female	No	R	3.5	9.4	5.9	27.4	Tt
Kousalya	114340	65	Female	No	R	3.3	9.6	6	30.2	TT
Kannayiram	112480	65	Male	No	F	3.1	9.1	5.4	28.3	Tt
Anandhi	118392	26	Female	No	R	3.5	9.1	5.2	27.8	TT
Nalini	117163	33	Female	No	F	3.1	9.2	4.9	27.1	TT
Loganadhan	108171	33	Male	No	F	3	9.3	4.7	28.1	TT
Anumandha Rao	108351	27	Male	YES	R	5.4	9.1	5.6	29.5	tt
Ganesh Kumar	94919	29	Male	No	F	3.4	9.5	5.2	28.7	Tt
Kannammal	115929	60	Female	No	F	3.8	9.6	5.8	27.9	Tt
Soliappan	113552	35	Male	No	R	3.7	9.7	4.2	29.9	Tt
Palani	105826	32	Male	No	R	3.1	9.2	4.8	30.4	tt
Pattabi raman	103563	63	Male	No	F	3.9	9.6	4.7	32.8	Tt
Mala	113646	40	Female	No	R	3.3	9.1	5.2	33.4	Tt
Subramani	104564	59	Male	No	R	3.2	9.5	4.1	31.2	TT
Ravi	101055	45	Male	No	F	3.5	9.1	5.6	33.2	TT
Syed Sukur	103416	43	Male	No	R	3.3	9.9	4.2	31.5	tt
Prema latha	113550	21	Female	No	R	3.4	9.9	4.4	34.3	Tt

MASTER CHART – CONTROL

NAME	AGE	SEX	24 Hr URINARY CALCIUM(mg/Kg/day)	SERUM CALCIUM(mg/dl)	SERUM PHOSPHORUS(mg/dl)	SERUM VITAMIN D3(pg/ml)	Taq 1 GENOTYPE
Devaraj	65	Male	3.4	10.2	4.9	32.2	Tt
Velmurugan	21	Male	3.2	10.1	4.2	33.2	TT
Ramanadhan	35	Male	6	9.8	4.6	33.5	Tt
Malliga	40	Female	3.2	10.4	5.4	31.6	Tt
Chitra	28	Female	2.8	9.7	6	36.4	TT
Mohan	26	Male	3.1	9.3	4.1	31.3	TT
Gangammal	50	Female	3.6	10.1	5.2	34.4	TT
Geethapriya	24	Female	3.4	10.2	4.6	36.6	TT
Kumar	31	Male	3.1	9.1	5.3	39.4	TT
Venkatammal	65	Female	3.8	9.7	4.4	27.3	TT
Margarette	45	Female	4.8	9.4	5.1	27.4	Tt
Kirubakar	55	Male	3.1	10.2	4.8	27.1	TT
Thangam	42	Female	3.2	9.8	4.7	28.5	Tt
Pasupathi	46	Male	2.8	9.7	4.5	27.6	Tt
Jayalakshmi	36	Female	3	9.4	4.4	27.8	TT
Sivalingam	53	Male	3.2	9.2	4.6	27.9	TT
Saraswathy	42	Female	3.4	9.4	5.3	27.4	TT
Perumal	39	Male	6.2	9.2	5.2	27.6	Tt
Kanaga	40	Female	3.1	9.4	5.6	29.6	TT
Sulochana	66	Female	3.8	10.1	4.1	34.3	Tt
Prabakaran	25	Male	2.7	10.4	5.4	28.5	TT
Masilamani	60	Male	3.4	9.6	4.9	29.1	Tt
Palani	55	Male	3	10.2	4.8	27.1	Tt
Kamala	55	Female	3.6	9.8	4.7	28.5	Tt

NAME	AGE	SEX	24 Hr URINARY CALCIUM(mg/Kg/day)	SERUM CALCIUM(mg/dl)	SERUM PHOSPHORUS(mg/dl)	SERUM VITAMIN D3(pg/ml)	Taq 1 GENOTYPE
Valliammal	49	Female	3.2	9.7	4.5	27.6	TT
Neela	34	Female	3.4	9.5	5.2	28.7	TT
Masi	54	Male	2.9	9.6	5.8	27.9	TT
Shanthi	40	Female	3.2	9.7	4.2	29.9	TT
Kannammal	52	Female	3.6	9.2	4.8	30.4	TT
Muniyammal	54	Female	3.8	9.6	4.7	32.8	TT
Venkatesan	64	Male	3.4	9.1	5.2	33.4	TT
Jeya	45	Female	3.6	9.5	4.1	31.2	Tt
Duraikannan	60	Male	3.2	9.1	5.6	33.2	Tt
Raj Kumar	37	Male	3.5	9.4	5.9	27.4	TT
Kokila	40	Female	3.3	9.6	6	30.2	Tt
Babu	28	Male	3.1	9.1	5.4	28.3	Tt
Nagabushanam	56	Female	3.5	9.1	5.2	27.8	TT
Prem kumar	33	Male	3.1	9.2	4.9	27.1	TT
Banumathy	33	Female	3	9.3	4.7	28.1	Tt
Siva kumar	32	Male	3.4	9.1	5.6	29.5	TT
Nandagopal	41	Male	3.4	9.5	5.2	28.7	TT
Naganmmal	60	Female	3.8	9.6	5.8	27.9	TT
Gopal	50	Male	3.7	9.7	4.2	29.9	Tt
Kanagavalli	50	Female	3.1	9.2	4.8	30.4	TT
Murugan	63	Male	4.9	9.6	4.7	32.8	tt
Gnanambigai	21	Female	3.3	9.1	5.2	33.4	TT
Arumugam	59	Male	3.2	9.5	4.1	31.2	TT
Venkatachalam	45	Male	3.5	9.1	5.6	33.2	TT
Swaminadhan	38	Male	3.3	9.9	4.2	31.5	Tt
Amudha	27	Female	3.4	9.9	4.4	34.3	TT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INTRODUCTION
Urinary tract stone formation is multifactorial. Alteration in metabolism are found in 40%-60% of stone formers. Among them idiopathic hypercalciuria is the most frequent.
Idiopathic hypercalciuria involves
An increase in intestinal calcium absorption
Enhanced demineralization of bones
1,25 $(OH)_2$ D_3 regulates target tissue biological response through genomic events which involves steroid hormonal intra cellular Vitamin-D receptor.
Researches has proven that increased calcium absorption is mediated by an increase in number of Vitamin-D receptors in intestine of genetic
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~ 1 ~ INTRODUCTION Urinary tract stone formation is multifactorial. Alteration in metabolism are found in 40%-60% of stone formers. Among them idiopathic hypercalciuria is the most frequent. Idiopathic hypercalciuria involves ? An increase in intestinal calcium absorption ? Enhanced demineralization of bones 1,25 (OH)2 D3 regulates target tissue biological response through genomic events which involves steroid hormonal intra cellular Vitamin-D receptor. Researches has proven that increased calcium absorption is mediated by an increase in number of Vitamin-D receptors in intestine of genetic hypercalciuric rats. Very little is known about genetic factors that mediate susceptibility to calcium...